Classical Hodgkin Lymphoma in Chinese and Dutch populations : epidemiology, EBV and HLA associations

Huang, Xin

University of Groningen

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Xin Huang

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Classical Hodgkin Lymphoma in Chinese and Dutch populations: Epidemiology, EBV and HLA associations

1. Classical Hodgkin Lymphoma (cHL) is a very heterogeneous disease.
2. The incidence of cHL is age dependent and both the overall incidence and the age dependent incidence are influenced by ethnic origin.
3. Immune control of Epstein Barr virus (EBV) infection depends on polymorphisms within the Human leukocyte Antigen (HLA) genes.
4. EBV-positive cHL is different from EBV-negative cHL in terms of epidemiology, association with ethnic origin and genetic predisposition.
5. The increased risk of individuals carrying the HLA-A*01 allele to develop EBV-positive cHL presumably relates to a less effective immune response against EBV derived peptides.
6. HLA-A*02:07 is an ethnic-specific HLA-A*02 allele variant that is related to a higher risk to develop EBV-positive cHL in the Chinese population.
7. I dreamed a thousand new paths. . . I woke and walked my old one.
8. Be not afraid of growing slowly; be afraid only of standing still.
9. A book holds a house of gold.
10. Even Homer sometimes nods (智者千虑, 必有一失).

Xin Huang

Promotores:	Prof. dr. J. H. M. van den Berg
	Prof. dr. S. Poppema
Copromotores:	Dr. A. Diepstra
	Dr. B. G. Hepkema
	Dr. I. M. Nolte
Beoordelingscommissie:	Prof. dr. C. J. M. Melief
	Prof. dr. A. B. Rickinson
	Prof. dr. E. Vellenga

Paranimfen: Wierd Kooistra

Yan He

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Chapter 1

Introduction and Scope of this thesis

Contents for Chapter 1

1. 1 General introduction on classical Hodgkin lymphoma

Hodgkin lymphoma(HL) is a distinct malignancy of the human lymphoid system and was described for the first time in 1832. It has an incidence of~5 per 100, 000 inhabitants in Western Europe and USA, but is less common in Asia with an incidence of <1 per 100, 000(1-3). HL can occur at any age, but is more frequent in young adults and elderly(4, 5). HL comprises two clinicopathological entities, namely nodular lymphocyte predominant HL(NLPHL) and classical HL(cHL)(6, 7). The latter is further subdivided based on the morphology of tumor cells and the composition of the background into four subtypes, i. e. nodular sclerosis HL (NSHL), mixed cellularity HL (MCHL), lymphocyte-rich HL and lymphocyte-depletion HL(6). This thesis focuses on cHL, which accounts for about 95% of all HL cases. CHL is characterized by a unique morphology in which the major part of the tumor mass is made up of various inflammatory cells, while the neoplastic cells, termed Hodgkin Reed-Sternberg(HRS) cells account for less than 1% of the cells. The HRS cells are identified as large, sometimes bi-nucleated cells with prominent nucleoli and an unusual/CD45-, CD30+, CD15+/-immunophenotype(9). The lineage of HRS cells has been enigmatic for a very long time. After successful introduction of the single cell isolation technology approximately two decades ago, HRS cells were proven to be of germinal center B-cell origin based on presence of immunoglobulin gene rearrangements with a high load of somatic mutations(8, 9).

Although the exact pathogenesis of cHL is still unknown, there is mounting evidence that a complex interplay of environmental and genetic factors contributes to the neoplastic development(10). Familial clustering of cHL and a 3~7 fold increased risk for cHL observed in siblings(11, 12) indicates that certain inherited traits increase the risk of developing cHL. A major environmental factor

is the Epstein-Barr virus(EBV) which is present in the HRS cells in a considerable proportion of cHL cases(13).

1. 2 Epstein Barr virus(EBV)

The Epstein-Barr virus (EBV), also called human herpesvirus 4(HHV-4), is a B lymphotropic γ-herpes virus that establishes a lifelong infection in > 90% of the total population worldwide, usually without causing symptoms (14). EBV is an enveloped virus with a 172-kb double-stranded DNA genome. Two EBV strains have been described: EBV-1 and EBV-2 (15). These two variants differ in the sequence of genes coding for the EBV nuclear antigens(EBNA-2 and EBNA-3) as well as in their efficiency to transform B-cells *in vitro*. The EBV-2 strain is less efficient in transforming B cells than the EBV-1 strain (15). Epidemiological studies demonstrated that the prevalence of theses two strains varies by geography. EBV-1 prevails in Europe, America and Asia, while EBV-2 is predominant in Africa(16). The contribution of the different EBV strains to specific diseases is still not known, but it has been found that the virus variant that prevails in a certain geographic region is also predominant in the EBV-related diseases(17).

The only known reservoir for infectious EBV is in the oropharynx and EBV is transmitted by saliva. Besides being the causal agent of infectious mononucleosis (IM), EBV is directly implicated in the pathogenesis of several carcinomas and malignant lymphomas, including nasopharyngeal carcinoma (NPC), Burkitt lymphoma(BL), cHL and lymphoproliferative disorders(LPD) arising in immuno-compromised individuals(18, 19). EBV can establish a productive(lytic) infection within its host cells, but its lifelong persistence depends on a nonproductive(latent)cycle, in which six EBV nuclear antigens(EBNA-1, -2, -3A, -3B, 3C and LP) and three membrane proteins (LMP-1, -2A and -2B) can be detected(19). All EBV-associated malignancies involve the virus's latent cycle,

although in most tumors some of the EBV latency associated genes are not expressed(18). Three different types of latency have been established. Type I latency is generally found in BL with a predominant expression of EBNA1. Type II latency, as seen in NPC and cHL, is characterized by expression of EBNA1 and two membrane proteins(LMP-1 and -2A). In Type III latency, all latency genes are expressed. Besides the latency type specific genes, EBERs(EBER-1 and -2) and BARF0 are usually also expressed in all types of latency.

LMP1 and LMP2a, the two membrane proteins exclusively expressed by the HRS cells of EBV+ cHL are well known for their ability to rescue pre-apoptotic germinal center B-cells, by mimicking CD40 and BCR signaling pathways (20) thereby contributing to or causing cHL development.

1. 2. 1 Immune control of EBV infection

In healthy carriers, EBV infection is under the control of strong host immune surveillance. Only some individuals develop cancer in spite of the strong oncogenic potential of EBV(21). Moreover, the observation of a high frequency of EBV-associated cHL and other EBV associated B-cell non-Hodgkin lymphomas in immunocompromised or immunodeficient individuals (22) suggests that a defective immunity results in an impaired EBV-specific immunosurveillance and permits tumor development. It has been well-established that cellular immune responses are mandatory for protective immunity against EBV infection, consisting mainly of Natural Killer(NK) cell mediated lysis and T cell adaptive immune responses (23, 24). Especially CD8+ cytotoxic T-cell responses are considered to be of central importance in both limiting the primary infection and controlling the numbers of latently infected B cells (24, 25). To initiate an EBV-specific immune response, EBV-derived antigenic peptides need to be presented by HLA class I via the endogenous processing pathway(23). In addition, there is accumulating evidence that supports an essential role for HLA class II-restricted

CD4+ T cell immune responses in the efficient eradication of EBV-infected cells (26, 27).

1. 2. 2 Infectious mononucleosis

Infectious mononucleosis(IM) was first described in 1920, but only until the late 1960s EBV was identified as its cause(28). The epidemiological features of IM with regard to its incidence and age distribution are in line with that of EBV infection. In Western populations, IM predominantly occurs in adolescents and is one of the most common infectious diseases in this age group (28, 29). In contrast, in Oriental populations, IM is a rather rare disease and more often occurs in childhood(30, 31). The observation that IM and EBV+ cHL have an overlap in age peak has led to a linkage hypothesis between these two diseases. Indeed, an excess risk for EBV-associated cHL was found in people with a history of IM with a typical incubation period from IM to cHL of 4~ 5 years(32-34).

1. 2. 3 Hodgkin lymphoma and associations with geography, age and subtypes

The prevalence of EBV infection in cHL is known to be highly variable in different geographic regions and racial groups, ranging from 20% in western countries to nearly 100% in South-America. In Asia the percentage is around 60% (4, 5, 35, 36). The fact that EBV can only be detected in a proportion of cHL cases suggests that another infectious agent or a different pathogenic pathway is involved in the development of EBV negative cHL cases. Thus, EBV-associated and non-associated cHL may represent two distinct etiological entities (37). In different racial groups a high incidence peak of non-EBV-associated cHL is always present in young adults. However there is marked heterogeneity in the prevalence of EBV-associated cHL with respect to patients' age, gender, race, and histological subtype(21, 38). In Western countries, a first incidence peak

occurs in adolescence and in young adults(15~ 34 years) and a second peak in older adults(> <50 years). In contrast, in Oriental countries a first peak presents much earlier in childhood. A second incidence peak in the elderly is similar to western countries. A male predominance in the prevalence of cHL, especially in EBV-associated cases has been reported by numerous studies(4, 5). In addition, EBV-associated cHL has a strong association with the MC subtype.

1. 3 Human Leukokocyte Antigen associations

The genes encoding the human major histocompatibility antigen, also called human leukocyte antigen (HLA) encompass 7. 6 Mb of the short arm of chromosome 6 and can be subdivided into HLA-class I and class II genes. This region is the most gene dense locus in the human genome and some of its extremely polymorphic genes code for the antigen-binding grooves of multiple HLA molecules. These highly polymorphic genes and codominant HLA expression result in a great variation in the repertoire of antigenic peptides that can be presented to the immune system. HLA class I is constitutively expressed on the membrane of all nucleated cells and presents proteasome degraded endogenous peptides of 8-11 amino acids in length to CD8+CTLs. In contrast, HLA class II has a restricted pattern of expression and can be detected on the surface of professional antigen-presenting cells(dendritic cells, macrophages, B cells), activated T cells and activated endothelium. The HLA class II molecule binds and presents antigenic peptides of 11-17 amino acids in length to CD4+T cells via exogenous processing pathways.

The potential role of immune-dysregulation in the pathogenesis of human cancers in concert with the vital importance of the HLA system in the regulation of host immune responses have attracted special attention on HLA-based disease associations. Aberrant expression of classical and non-classical HLA class I and II molecules by tumor cells observed in a number of human

malignancies indicates that downregulation or complete loss of HLA molecules is a mechanism for tumor cells to escape from host immune surveillance(39-42). Moreover, certain HLA alleles have been reported to be associated with disease susceptibility and resistance in more than 40 different diseases(41, 42). Certain HLA alleles were found to be the most significant genetically determined factor in predicting a high or low risk for several common autoimmune diseases, such as HLA-B27 and ankylosing spondylitis(AS), HLA-DR1 or HLA-DR4 and rheumatoid arthritis (RA), HLA-DQ2 or HLA-DQ8 and celiac disease (CD) (43). With accumulating evidence supporting a role of host immunosurveillance in cancer development and progression, HLA and cancer associations attracted a lot of attention and have been investigated in different human malignancies. HLA-DQB1*0301 and HLA-DRB1*04051 were reported to confer an increased risk for gastric adenocarcinoma in Caucasians and Japanese respectively(44, 45). HLA-DQB1*0301 also was linked with genetic predisposition to melanoma in Caucasians (46). HLA-A*02 and HLA-A*11 were associated with genetic susceptibility or resistance to NPC in the Southern Chinese population.

1. 3. 1 HLA antigen expression in HRS cells

Expression of HLA class I and class II antigens by HRS cells in cHL have been investigated in the western population by several research groups (47-49). Downregulation or absence of membranous expression of HLA class I by HRS cells in cHL ranged from 30 to 40% of cases in different studies. Studies showed a positive correlation between HLA class I expression and EBV status in cHL. Downregulation or lack of HLA class II expression was observed in the HRS cells of 40~ 50% cHL. Diepstra et al(50) reported that HLA class II expression was closely correlated with positive EBV status. Although this association was not statistically significant in the study of Oudejans et al. , a trend to maintain the expression of HLA class II was observed in EBV+patients(49). Another interesting

finding was the negative association between HLA class II expression and response to first line therapy as well as relative survival in cHL reported by Diepstra et al(50).

1. 3. 2 HLA-linked genetic susceptibility in cHL patients

CHL was one of the first diseases for which an association was observed with HLA serotypes and the first description dates back to 1967(51). An important rationale for a particular interest in exploring HLA associations with cHL is its well-documented infectious etiology. As mentioned earlier, the immune control of EBV infection mainly depends on HLA-restricted T-cell responses. Moreover, it is well-known that different HLA alleles possess different abilities to bind and present EBV-derived antigenic peptides to effector cells. This indicates that the specificity of HLA alleles is an important determinant for the effectiveness of controlling EBV infection. Thus, certain HLA alleles may predispose to the development of EBV-related diseases.

An extensive literature search on the relationship between HLA and cHL susceptibility reveals that specific HLA serotypes and alleles can confer a decreased or increased risk for both familial and sporadic cHL in Caucasians(52-54). In a genetic screening approach using polymorphic microsatellite markers, two markers located in the HLA class I region were reported to be associated with EBV-positive cHL in a Dutch population (54). Subsequent studies demonstrated that two specific HLA-A alleles, HLA-A*01 and A*02 are associated with an increased and decreased risk for EBV-associated cHL respectively in a Dutch and Scottish population (55). Moreover, recent studies also proposed that individuals carrying the HLA-A*01 allele have an increased risk to develop IM(56), further substantiating the etiological linkage(EBV infection) between the two diseases.

In conclusion

Classical HL is a distinct malignancy of the lymphoid system with an unusual and complex morphology, epidemiology and etiology. It's evident that the HRS cells employ multiple strategies to escape from host immune surveillance and the quality of antigen presentation in the context of HLA might be a crucial pathogenic factor. Accordingly, multiple HLA associations with cHL have been reported. Moreover, associations specifically with EBV associated cHL strengthen the importance of HLA in cHL. The potential effect of HLA alleles on the outcome of EBV infection suggests a HLA-linked genetic susceptibility to EBV-associated cancers. Furthermore, because of the highly polymorphic nature of the HLA system and its ethnic linkage, the HLA associations may explain the epidemiological heterogeneity of this disease. In addition, it might also provide an opportunity for the generation of virus-specific HLA-restricted T-cells to be used in immunotherapeutic approaches.

1. 4 The scope of this thesis

This thesis focuses on the comparison between cHL patients with a different ethnic background for the association of EBV, HLA antigen expression as well as HLA-based immunogenetics. A description on epidemiological features of 371 cHL patients from Northern China diagnosed during 1997 ~ 2008 is given in chapter 2. In chapter 3, for the first time, the expression of HLA antigens by HRS cells in association with EBV status is shown in a group of Chinese cHL patients. Since the highly prevalent HLA-A*02 allele was found to be protective against EBV+ cHL in a Dutch population, we next studied the significance of this allele in Chinese cHL patients in chapter 4. In chapter 5, a comprehensive screening of all classical HLA alleles was carried out using DNA samples from 338 Dutch cHL patients to further define the most relevant candidate genes or SNPs associated with(EBV-associated) cHL.

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Chapter 2

Epidemiology of Classical Hodgkin Lymphoma and its Association with Epstein Barr Virus in Northern China

Xin Huang[1, 2], Ilja Nolte[3], Zifen Gao[2], Hans Vos[1], Bouke Hepkema[4], Sibrand Poppema[1], Anke van den Berg[1] and Arjan Diepstra[1]

[1]Department of Pathology and Medical Biology, [3]Unit of Genetic Epidemiology and Bioinformatics, Department of Epidemiology and [4]Department of Laboratory Medicine, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands. [2]Department of Pathology, Health Science Center, Peking University, Beijing, China.

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Abstract

Background: The incidence of classical Hodgkin lymphoma (cHL) and its association with Epstein-Barr virus (EBV) varies significantly with age, sex, ethnicity and geographic location. This is the first report on epidemiological features of cHL patients from Northern China. These features are compared to data from a previously published Dutch cHL population.

Methodology/ Principal Findings: 371 cHL patients diagnosed between 1997 and 2008 in the North of China were included. EBV status was determined by immunohistochemistry for latent membrane protein 1 and/or in situ hybridization of EBV- encoded small RNAs. The Dutch population-based cohort included 515 cHL patients diagnosed between 1987 and 2000. In the Chinese population, tumor cells of 41% of the cHL patients were EBV+and this was significantly associated with male sex, mixed cellularity subtype and young age(<20y). Median age of Chinese patients was 10 years younger than that of Dutch patients(27y vs 37y). In addition, the age distribution between the two populations was strikingly different in both the EBV+subgroups($p=1.8\times10^{-8}$) and the EBV- subgroups($p=1.8\times10^{-4}$). Chinese patients had a higher male-to-female ratio than Dutch patients(2.1 vs 1.4 in all patients and 3.3 vs 1.9 in the EBV+subgroup).

Conclusion/Significance: CHL patients from Northern China show a distinctive age distribution pattern with a striking incidence peak among children and young adults. In comparison to Dutch cHL patients there are pronounced differences in age distribution, sex, subtype and EBV status, presumably caused by complex gene-environmental interactions.

Introduction

Classical Hodgkin lymphoma (cHL) is a heterogeneous malignancy with a complex etiology and epidemiology. In general, cHL accounts for about 1% of all cancers and ~30% of the lymphoid malignancies worldwide (1). Epidemiologic studies of cHL demonstrate a remarkable diversity of the incidence according to age, sex, ethnic background, geographic location and socioeconomic status(2, 3). The highest incidence was reported among Caucasians, followed by African Americans and Hispanics, and the lowest incidence was found in Orientals (3). Data from the International Agency for Research on Cancer (http://globocan. iarc. fr/) shows a nearly 6-fold difference between Western Europe and East Asia with an incidence of 2. 3 and 0. 4 per 100, 000 inhabitants per year respectively in 2008. A genetic explanation for this difference has been shown in a multi-ethnic study of cHL in the United States that reported the lowest incidence rate in Asian immigrants in comparison to other ethnic origins (4). However, a trend of increasing incidence of HL was reported among Chinese immigrants in western countries(4, 5), suggesting an influence of westernization. The incidence pattern by age was also shown to be different between Caucasian and Oriental populations(6, 7). Western populations typically have a bimodal age distribution with two peaks near 25 and 60ys. In Orientals, a first incidence peak usually presents in childhood with a second peak in the elderly, although in Japanese cHL patients the early incidence peak was reported to be absent(4, 8).

In a proportion of cHL patients, Epstein-Barr virus (EBV) is present in the tumor cells and EBV is accepted as a causal agent in these patients(7, 9). There is a striking variability in the percentage of EBV involved cases between racial groups and geographic locations (2, 10). The proportion of EBV involvement is almost 100% in Hispanic cHL patients(11), much lower in Caucasians(20~40%) (2, 12) and intermediate in Orientals(13, 14). In general, the EBV association with

cHL is related to age, being the strongest in children and the elderly(15, 16). In addition, male sex and the mixed cellularity (MC) histological subtype are associated with EBV+cHL worldwide(15, 17).

The current study was undertaken to investigate the epidemiological characteristics of cHL patients from Northern China. In addition, these characteristics were compared to data from a previously reported Dutch cHL patient population(18).

Materials and Methods

Patient selection and data collection

371 cHL patients, of whom sex, age, histological subtype and EBV status were known, were included in this study(19). These patients resided in the Northern area of China and were diagnosed with cHL during the period of 1997 to 2008 at the Dept. of Pathology, Health Science Center, Peking University. 198 of these cHL patients were diagnosed and treated in the affiliated hospitals of Peking University. The other 173 patients were submitted for consultation to confirm the diagnosis and were mainly from other hospitals in Beijing City and the hospitals of its neighboring city or provinces including Tianjin City, Hebei Province, Shanxi Province and Jilin Province. For all patients we retrieved the original data including patients' sex, age, histological subtype and tumor cell EBV status at the time of diagnosis from the pathology database. Ethical approval for this study was not required by these institutions as the experiments carried out did not relate to patients privacy or treatment. Research was conducted adhering to the Declaration of Helsinki and according to Dutch regulations (http://www. federa. org/). The institutional review board (the Medical Ethics Review Board of the University Medical Center Groningen) specifically waived the need for ethics approval.

Histopathological reclassification

Haematoxylin and eosin-stained slides from 157 formalin fixed paraffin embedded tissue blocks were available for histological reclassification according to the most recent WHO classification system (1) which categorizes cHL into four histological subgroups, i. e. nodular sclerosis (NS), mixed cellularity (MC), lymphocyte rich(LR) and lymphocyte depletion(LD). Cases without enough tissue for proper evaluation of the background architecture were designated as cHL not otherwise specified(NOS).

EBV status

For 246 cases latent membrane protein 1 (LMP1) staining was performed by immunohistochemistry (IHC) in a routine diagnostic setting to determine the presence of EBV using the CS1-4 antibody(DAKO, Glostrup, Denmark). For 236 cHL cases, presence of EBV in tumor cells was determined by EBER in situ hybridization(ISH) with a fluorescein-conjugated PNA probe specific for the EBV-encoded EBER RNAs (DAKO, Glostrup, Denmark) using standard laboratory protocols. Appropriate positive and negative controls were included in all analysis.

Statistical analysis

Differences between EBV + and EBV-groups in relation to age, sex and histopathological subtype were assessed by Chi square test, Fisher's exact test or Mann Whitney U test. The data were analyzed with SPSS for Windows, version 17. 0. A p-value<0. 05 was considered significant.

Results

Detection of EBV

IHC for LMP1 was performed in 246 patients and was positive in 32%. ISH for EBERs was carried out in 236 cHL patients and revealed presence of EBV in 44%. For 111 cases EBV status was determined with both methods and the concordance rate was 87. 4% (Table 1). In the 14 discrepant cases, 12 were negative for LMP1 and positive for EBER and 2 were negative for EBERs and positive for LMP1. Since EBER ISH has been widely accepted as the golden standard for detection of EBV latent infection, we used the results of the EBER ISH for these 14 cases. Taking the results of the EBER ISH(n=236) together with those of the cases that were analyzed only by LMP1 staining(n=135) revealed EBV positivity in 41%(n=152) of patients.

Table 1 EBER ISH and LMP-1 IHC in Chinese cHL patients

	EBER+	EBER-	Total
LMP1+	30	2	32(28. 8%+)
LMP1-	12	67	79
Total	42(37. 8%+)	69	111

Histopathological reclassification

157 cHL cases were re-evaluated for histological classification of the subtype. The histological subtype could not be unequivocally determined in 29(18%) cases and was designated cHL-NOS. Six and 9 of these cases were previously classified as LR and MC subtype respectively. Discrepancies in the classification were observed in 5 out of 13 LR cases and 6 out of 57 MC cases which were reclassified as MC and NS subtype respectively. All original NS diagnoses were

reclassified as NS subtype. A consistent classification was achieved for 117 of 128(91. 4%) cHL cases.

In the total Chinese patient population, NS was the most common subtype, accounting for 48% of cases(n=161), followed by MC with 39% (n=130), LR with 12% (n=40) and LD with 0. 3% (n=1) of cases(Table 2).

Distribution of age, sex and histological subtypes

The male to female ratio was 2. 1:1 in the total group, 3. 3:1 in the EBV+ group and 1.6mm: 1 in the EBV- group (p=0. 001). The MC subtype (63%) was more common in EBV+cHL patients and the NS subtype(69%) was more common in the EBV- group($p=1.9\times10^{-20}$). The median age of cHL patients in the EBV+group was similar to that of the EBV- cHL patient group (27 years, range:3-76ys and 27 years, range:6-75ys respectively)(Table 2). However, comparison of the 10 years age group distribution between EBV + and EBV- patients did show a highly statistically significant difference ($p=2.6\times10^{-12}$) (Figure 2). Patients in the age groups from 0 to 20 and from 70 to 80 years of age were significantly more often EBV+, while patients aged 20 to 30 were significantly more often EBV-.

Table 2 Distribution of age,sex and histology by EBV status in Chinese cHL patients

	Reclassified		Not reclassified		All patients		EBV+		EBV-		EBV+ vs EBV-
	n= 157		n= 214		n= 371		n= 152		n= 219		P
	n	%	n	%	n	%	n	%	n	%	
Sex											
Male	101	64	149	70	250	67	117	77	133	61	0.001*
Female	56	36	65	30	121	33	35	23	86	39	
Histological subtype											
NS	80	62.5	81	39.7	161	48	24	18	137	69	1.9×10^{-20}†
MC	46	35.9	84	41.2	130	39	83	63	47	24	
LR	2	1.6	38	18.6	40	12	27	20	13	7	
LD			1	0.5	1	0.3			1	0.5	
NOS	29		10		39		18		21		
Median age(range)	28 (4~ 74)		27 (3~ 76)		27 (3~ 76)		27 (3~ 76)		27 (6~ 75)		0.298‡

* Chi square test,† Fisher's exact test, NOS cases were excluded,‡Mann Whitney U test, NS indicates nodular sclerosis; MC, mixed cellularity; LR,lymphocyte rich; LD Lymphocyte depletion; NOS,not otherwise specified

Comparison between Chinese and Dutch cHL patients

The median age of Chinese patients(27ys, range: 3-76ys) was 10 years younger than that of the Dutch population(37ys, range: 8-94ys)($p=1.3\times10^{-11}$) and the age distribution in 10-year intervals was significantly different($p=1.3\times10^{-11}$)(Figure 1, Table 3). Similar to the Chinese patients, stratification by EBV status showed significant differences in median age between EBV+(43 years, range: 8-94) and EBV- (33 years, range: 9-90) Dutch cHL patients ($p=2.4\times10^{-5}$). Comparison of the EBV+Chinese patient group with the EBV+ Dutch patient group showed a striking difference in the age distribution ($p=1.8\times10^{-8}$)(Figure 2A, Table 3). Also the age distribution of the EBV- Chinese patients was significantly different from the EBV- Dutch patients($p=1.8\times10^{-4}$)(Figure 2B, Table 3).

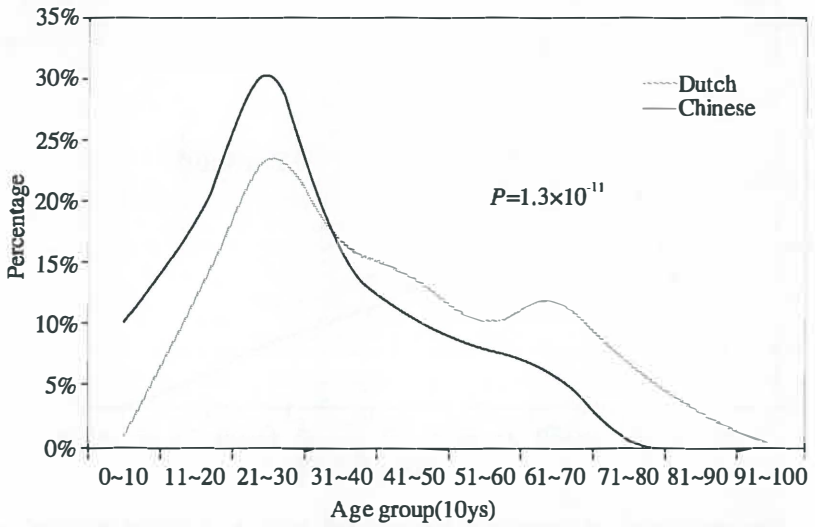

Figure 1 **Age distribution of Dutch and Chinese cHL patients in consecutive 10-year intervals.** Age distribution is shown for 371 patients from Northern China(black) and 515 patients from the Northern part of the Netherlands (grey). A significant difference was observed in the age distribution between these two patients groups ($p=1.3\times10^{-11}$). These two curves exhibit a somewhat similar trend with apparent differences at the first and sixth decades between the Chinese and the Dutch population (9% vs 1% and 7% vs 12% respectively). The Chinese population has more patients among the first three decades as compared to the Dutch population, both having the highest peak in the third decade. The Chinese population does not show a second incidence peak at around 60 years.

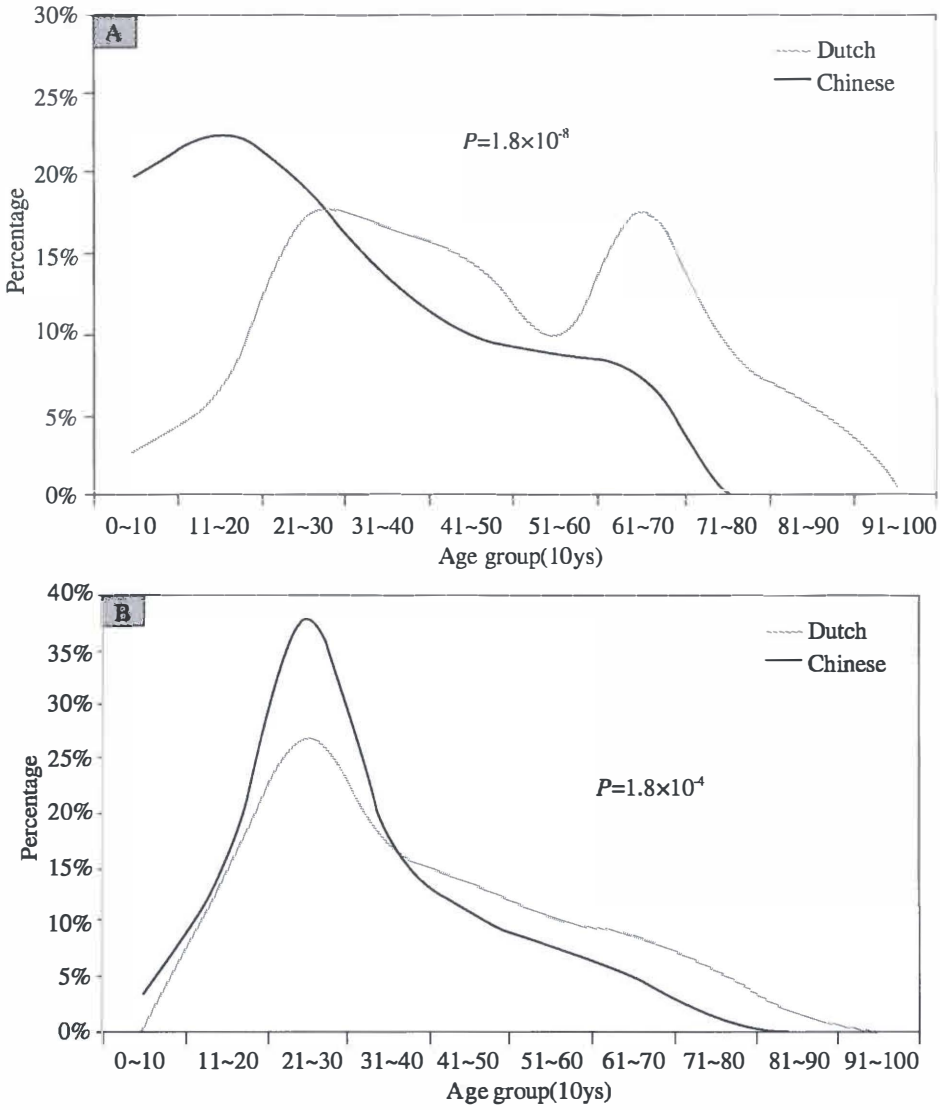

Figure 2 **Age distribution of Dutch and Chinese cHL patients stratified by EBV status.** A represents the comparison of the age distribution in the two EBV+cHL subpopulations. Chinese EBV+cHL patients(black) exhibited a prominent single peak in the first and second decade with a maximum percentage of 24%. In contrast, Dutch EBV+cHL patients(grey) demonstrated a clear bimodal age distribution pattern with two peaks occurring in the third and seventh decade respectively with a similar incidence for both peaks (17% and 18%). B shows the age distribution curve of the EBV- cHL patients. There is a single incidence peak in the third decade for both populations, which is more pronounced in the Chinese population(38% vs 27%).

Table 3 Differences in age distribution in Chinese and Dutch EBV positive and negative patients

Chinese Dutch

	Chinese			Dutch			p-value
	N	Age		N	Age		
		Median	Range		Median	Range	
All patients	371	27	3-76	515	37	8-94	1.3×10^{-11}
EBV-	219	27	6-75	334	34	9-90	1.8×10^{-4}
EBV+	152	27	3-76	181	43	8-94	1.8×10^{-8}

A significant difference in the sex distribution between Dutch and Chinese populations was found in the total cHL patient group with a male to female ratio of 2.1:1 in the Chinese and 1.4:1 in the Dutch population(p=0.0036). This difference was more pronounced in the EBV+group where males were overrepresented in the Chinese as compared to the Dutch population(3.3:1 vs 1.8:1)(p=0.016), whereas in the EBV- group there was no significant difference. The differences in the male to female ratio in the Dutch population were less pronounced(1.4:1 in the total, 1.2:1 in the EBV- and 1.8:1 in the EBV+ group) as compared to the Chinese population(Table 4).

Table 4 Differences in sex distribution in Chinese and Dutch EBV positive and negative patients

	Chinese			Dutch			P-value
	Male	Female	ratio	male	Female	Ratio	
All patients	250	121	2.1 : 1	297	218	1.4 : 1	0.0036
EBV+	117	35	3.3 : 1	118	64	1.8 : 1	0.016
EBV-	133	88	1.5 : 1	180	154	1.2 : 1	NS

To rule out a possible bias in the Chinese cHL population caused by determining the EBV status only by LMP1 staining, we also compared the distribution of age and sex in the Dutch population with that of the 236 Chinese patients analyzed by EBER-ISH. The results of this comparison were comparable

to those of the total Chinese patient group(data not shown).

Discussion

Epidemiological studies on cHL have shown significant variation in age and EBV distribution in relation to ethnic background and geographic location. Asians are known to have the lowest incidence of cHL and an intermediate proportion of EBV-associated cHL(3). Previously, a few studies reported on cHL epidemiology in Taiwan and Hong Kong, populations that are closely related to the Southern Chinese population (13, 20-22). Importantly, the Northern Chinese population is genetically different from the Southern Chinese population, especially in the distribution of Human Leukocyte Antigen(HLA) types. Undifferentiated nasopharyngeal carcinoma (UNPC), another EBV-associated malignancy with a latent EBV expression pattern similar to EBV+cHL, is endemic in Southern China but not in Northern China. Previous studies proposed that the geographic-restricted distribution of the UNPC susceptibility allele HLA-A*02:07 might well explain the high incidence of UNPC in the Southern Chinese population. Thus, differences in cHL incidence and epidemiology might also be expected in different Chinese regions. The main aim of this study was to analyze the epidemiology of cHL in the Northern part of China, a population that has not been studied before.

Previous studies found that cHL was EBV+at a frequency of 65%(n=23) and 61%(n=28) by EBER-ISH and 63%(n=70) by combining the results of LMP1 IHC and EBER ISH in cHL patients from Hong Kong, China and Taiwan respectively (13, 14, 21). These frequencies are higher than the 41% of EBV positivity identified in the present patient group. However, the patient cohort size in the current study(n=371) was much larger than these previous studies. The difference in EBV positivity was only marginally influenced by determination of EBV status by LMP1 IHC in a subset of patients. EBER ISH only slightly increased the percentage of EBV positivity from 41% to 44%. Besides the population size,

differences in EBV prevalence might also be influenced by intra-ethnic environmental and genetic variations. Consistent with previous findings in Chinese and other ethnic groups of cHL patients, the incidence of EBV+cHL was significantly higher in males and the MC subtype(2).

A shift in the histological classification upon histopathological re-examination was observed in 8. 6% (n = 11) of the cases, partly explained by changes in the WHO classification guidelines. Agreement was specifically poor for the LR and MC subtypes. Due to the lack of enough tissue for evaluating the background architecture, 15 cases previously diagnosed as LR or MC subtype were re-assigned as cHL-NOS. Overall, the histological distribution was similar to those reported in previous Chinese populations from Hong Kong and Taiwan(13, 23).

Until now, there are four studies that investigated the age distribution of cHL in the Chinese population (13, 20-22). Three studies, including two from Hong Kong(n=23 and n=92)(13, 20) and another one from Taiwan(n=70)(21), identified a bimodal age distribution with two peaks at the second and sixth decades respectively, whereas another study from Taiwan(n=42)(22) found a single age peak at the 3rd decade. Only consistent with this last study, we also found a unimodal age distribution with a distinctive peak incidence occurring at the 3rd decade. Only two of the four studies (Hong Kong and Taiwan) included EBV status into the analyses(13, 21). Our results were consistent with these studies for EBV- cHL patients with a single incidence peak in young-adults. However, our EBV+cHL patients did not show an incidence peak in the elderly, in contrast to both other studies.

Comparison of the Chinese and Dutch cHL populations revealed an inter-ethnic significant difference in the age distribution in the total patient groups and also in the EBV+and EBV- subgroups separately. A bimodal pattern for the age distribution of cHL in general as well as in the EBV + subgroup is considered

typical for the Western population and this was also present in our Dutch cohort. It has been reported that in the Asian cHL population, an early peak should be less obvious and at a younger age compared to the Western population, whereas a second peak at older age should be similar (4, 8). Intriguingly, our Chinese population had an evident early peak similar to the Dutch population but lacked the peak at older age. It is unlikely that this could be the result of an age dependent selection bias in the Chinese population.

According to the literature, EBV+Asian cHL shows a similar age distribution as EBV- Asian cHL. However, in the present study EBV+cHL had a different and pronounced incidence peak at adolescence. In both the Chinese and the Dutch populations a clear peak for the EBV- patients was observed at the 3rd decade. Remarkably, this peak was higher in the Chinese population than in the Dutch population(38% vs 27%).

An inter-ethnic difference was also observed for the sex distribution of all patients and of the EBV+cHL patients. In general, the Chinese population had a higher male-to-female ratio than the Dutch population (2. 1 vs 1. 4) and this difference was even more prominent in EBV + cHL patients (3. 3 vs 1. 9) and virtually absent in EBV- cHL patients(1. 5 vs 1. 2).

In conclusion, we demonstrate a distinctive age distribution pattern in by far the largest reported group of Northern Chinese cHL patients with a striking incidence peak among young adults. Moreover, we identified large inter-ethnic differences in the distribution of age, sex, EBV status and histological subtype in comparison to a Caucasian population. Both genetic and environmental factors are expected to play a role in these differences.

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Chapter 3

Expression of HLA Class I and HLA Class II by Tumor Cells in Chinese Classical Hodgkin Lymphoma Patients

Xin Huang[1, 2], Anke van den Berg[1], Zifen Gao[2], Lydia Visser[1], Ilja Nolte[3], Hans Vos[1], Bouke Hepkema[4], Wierd Kooistra[1], Sibrand Poppema[1] & Arjan Diepstra[1*]

1Department of Pathology and Medical Biology, 3Unit of Genetic Epidemiology and Bioinformatics, Department of Epidemiology and 4Department of Laboratory Medicine, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands. 2Department of Pathology, Health Science Center, Peking University, Beijing, China.

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Abstract

Background: In Caucasian populations, the tumor cells of Epstein Barr virus (EBV)-positive classical Hodgkin Lymphomas (cHL) patients more frequently express HLA class I and HLA class II molecules compared to EBV-negative cHL patients. HLA expression(in relation to EBV) in Asian cHL patients has not been previously investigated.

Methodology/Principal Findings: We randomly selected 145 cHL patients with formalin-fixed, paraffin embedded tissue blocks available from 5 hospitals from the Northern part of China. Hematoxylin & Eosin-stained slides were used to reclassify the histological subtypes according to the WHO classification. EBV status was determined by visualization of EBERs in tumor cells using in situ hybridization. Membranous expression of HLA molecules was detected by immunohistochemistry using antibodies HC-10(class I heavy chain) and anti- 2-microglobulin for HLA class I, and CR3/43 for HLA class II. EBV+tumor cells were observed in 40% (58/145) of the cHL patients. As expected, the percentage of EBV+cases was much higher in the mixed cellularity subtype(71%) than in the nodular sclerosis subtype (16%) ($p < 0.001$). Expression of HLA class I was observed in 79% of the EBV+cHL cases and in 30% of the EBV- cases ($p<0.001$). For HLA class II, 52% of EBV+cHL cases were positive, compared to 43% in EBV- cases($p=0.28$).

Conclusions: The results in the Northern China population were similar to those in the Caucasian population for HLA class I, but not for HLA class II.

Introduction

Classical Hodgkin lymphoma (cHL) is a malignant neoplasm of the immune system, characterized by a minority of B cell derived tumor cells, named Hodgkin Reed-Sternberg cells (HRS cells) and numerous reactive cells consisting of lymphocytes, histiocytes, eosinophils, and plasma cells. The HRS cells are large, sometimes bi- or multinucleated cells with prominent nucleoli and a characteristic CD20 negative to weakly positive, CD30+ and CD15+/- immunophenotype (1). The presence of HRS cells in an abundant inflammatory infiltrate indicates that anti-tumor immune responses apparently are insufficient for the eradication of HRS cells. It has been shown that the tumor cells of cHL employ several mechanisms to escape from immune responses, even more so in Epstein Barr virus (EBV) associated cases (2-4). EBV has been acknowledged as the major infectious agent causing cHL, although the proportion of EBV associated cHL varies from 20% to nearly 100% in different populations (3, 5). In addition, the proportion of EBV+ cases is also age-dependent with a first high incidence peak in children and a second peak in adults around age 60 (3, 5). EBV-infected HRS cells consistently express a limited set of proteins, consisting of latent membrane protein 1(LMP1), latent membrane protein 2(LMP2) and EBV nuclear antigen 1 (EBNA1) (5). Antigenic peptides derived from these three proteins can be processed and presented by the human leukocyte antigen(HLA) class I and class II pathways, the efficiency of which largely depends on the peptide binding affinity of the highly polymorphic HLA alleles (6-9). Cytotoxic T lymphocytes (CTLs) are known to be the primary effector cells to eradicate EBV-infected B cells that present LMP1 and LMP2 antigenic peptides in the context of appropriate HLA class I molecules (6, 7). In addition, there's *in vitro* evidence that EBV infection and the related malignant transformation are controlled by CD4 + T cells, depending on HLA class II restricted antigen presentation (10). In other words,

both HLA class I-restricted CTL responses and HLA class II-restricted CD4 +T-cell responses are essential for a successful anti-tumor immune defense. Therefore, downregulation of HLA class I and HLA class II antigens might be implicated in the pathogenesis of cHL by allowing tumor cells to escape host immunosurveillance.

Several research groups have studied the association between HLA expression and cHL in the Western population(11-16), but nothing is known for the Asian population. Since HLA types are known to widely differ between Caucasians and Asians, we set out to investigate the expression of HLA molecules in Chinese cHL cases for drawing comparison between the two populations. We studied HLA class I as well as HLA class II expression in relation to EBV status in a population from the Northern part of China.

Materials and Methods

Patient material

Formalin-fixed paraffin-embedded tissue blocks of lymph node biopsies from 145 cHL patients were obtained from 5 hospitals in northern China(Dept. of Pathology, Health Science Center, Peking University; Dept. of Pathology, First Hospital of Jilin University; Dept. of Pathology, Shougang Hospital, Peking University; Dept. of Pathology, Beijing Air Army General Hospital; Zhanye Regional Hospital, Gansu Province). The biopsies were stained with hematoxylin & eosin(H&E) and histopathological subtyping was performed according to the WHO classification.

In situ hybridization

Detection of EBV in tumor cells was performed by *in situ* hybridization(ISH) on paraffin sections with a fluorescein-conjugated PNA probe specific for the EBV-encoded EBER RNAs(DAKO, Glostrup, Denmark). A known EBV+tissue section

was used as a positive control.

Immunohistochemical staining

4- m thick paraffin sections were deparaffinized by xylene and rehydrated through a graded ethanol series into water. Microwave antigen retrieval was performed with Tris-EDTA solution (10mM Tris Base, 1mM EDTA Solution, PH 9. 0) and endogenous peroxidase activity was blocked in 3% H_2O_2. The expression of HLA class I was detected using monoclonal antibody HC-10 at a dilution of 1: 200 (kindly provided by Prof. dr. J. Neefjes, the Netherlands Cancer Institute, Amsterdam), which recognizes HLA B and C molecules, as well as a few HLA-A molecules (17). In addition, the polyclonal rabbit anti human 2-microglobulin (DAKO) at a dilution of 1:200 was used as an additional marker to detect HLA class I. For detection of HLA class II, we used the CR3/43 monoclonal antibody (DAKO) that binds to a specific monomorphic epitope in the β chain of HLA-DP, HLA-DQ and HLA-DR. All antibodies were detected using a standard Avidin Biotin Complex(ABC) immunoperoxidase method. Diaminobenzidine was used as the chromogen and hematoxylin was used for counterstaining.

Evaluation of HLA class I and class II staining

HLA class I heavy chain (HC-10) staining was scored simultaneously with 2-microglobulin staining. The same scoring rules were used for HLA class II. The surrounding inflammatory cells were used as an internal positive control and also as a reference for assessing the intensity of HLA expression by HRS cells. A strong membranous staining on at least 50% of the tumor cells was identified as positive. In case the staining intensity on the tumor cells was similar to the intensity on the surrounding reactive cells, membranes in between adjacent tumor cells were evaluated.

Statistical analysis

HLA expression was determined in relation to EBV status. Differences between EBV+ and EBV-neg groups in relation to HLA expression as well as several clinicopathologic variables were assessed by Chi square test, Fisher's exact test or Mann Whitney U test. The correlation between HLA class I and class II expression was evaluated with Chi square test. In addition, multivariate analysis using logistic regression was performed to adjust for confounders. The data were analyzed with SPSS for windows, version 16. 0. A p-value <0. 05 was considered significant.

Results

Clinicopathologic features

145 patients diagnosed with cHL were subdivided into histological subtypes according to the WHO classification. Subtype could not be unequivocally determined in 18% (n = 26), usually because there was not enough tissue to properly evaluate the background architecture. These patients were classified as cHL, not otherwise specified (NOS). In the remaining patients the nodular sclerosis(NS) subtype was the most common one, accounting for 63% (n=75) of patients, followed by mixed cellularity (MC) with 35% (n = 42) of patients. The lymphocyte rich(LR) subtype was rare(n=2) and the lymphocyte depleted subtype was absent. The median age of the patients at the time of diagnosis was 28 years, ranging from 4 to 74 years. There was a clear male predominance with a male to female ratio of 2:1.

EBV status and clinicopathologic variables

EBER staining showed consistent nuclear labeling in all HRS cells in 40% of the

patients(n=58)(see figure 1). A low number of positive small bystander cells were observed in some cases.

Subtype and sex demonstrated statistically significant differences between EBV+and EBV-neg cases. As expected, the MC subtype showed the highest percentage of EBV + cases (30 of 42 cases [71%]). In addition, males more frequently had EBV + cHL than females (48% compared to 24%). In terms of patients' age, no significant difference was found between EBV-associated and non-EBV-associated cHL(Table 1).

Table 1 Distribution of age, sex and histology by EBV status

	All patients n=145	EBV-Positive n=58	EBV-Negative n=87	P
median age (range)	28(4- 74)	31(4-74)	28(8-74)	0. 56‡
Sex				
Male	66. 2% (n=96)	79. 3% (n=46)	57. 5% (n=50)	0. 006*
Female	33. 7% (n=49)	20. 7% (n=12)	42. 5% (n=37)	
Histological subtype				
NS	51. 7% (n=75)	20. 7% (n=12)	72. 4% (n=63)	<0. 001†
MC	29. 0% (n=42)	51. 7% (n=30)	13. 8% (n=12)	
LR	1. 4% (n=2)	3. 4% (n=2)	0% (n=0)	
NOS	17. 9% (n=26)	24. 2% (n=14)	13. 8% (n=12)	

‡Mann Whitney U test, * Chi square test, †Fisher's exact test, NS indicates nodular sclerosis; MC, mixed cellularity; LR, lymphocyte rich; NOS, not otherwise specified

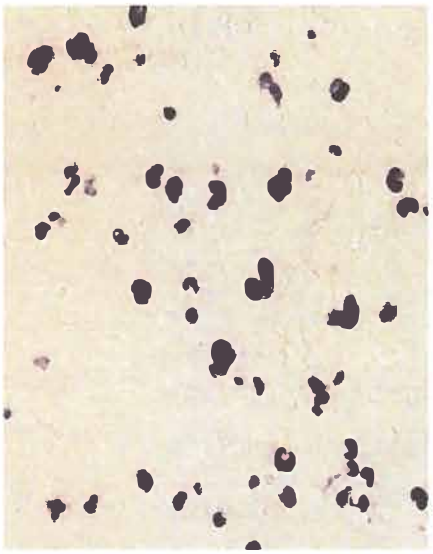

Fig 1 **In situ hybridization(ISH) for EBERs in an EBV+cHL case.** The EBER-ISH revealed a homogeneous positive signal in the nucleus of all Hodgkin and Reed-Sternberg(HRS) cells as well as in some small reactive cells (original magnification ×40).

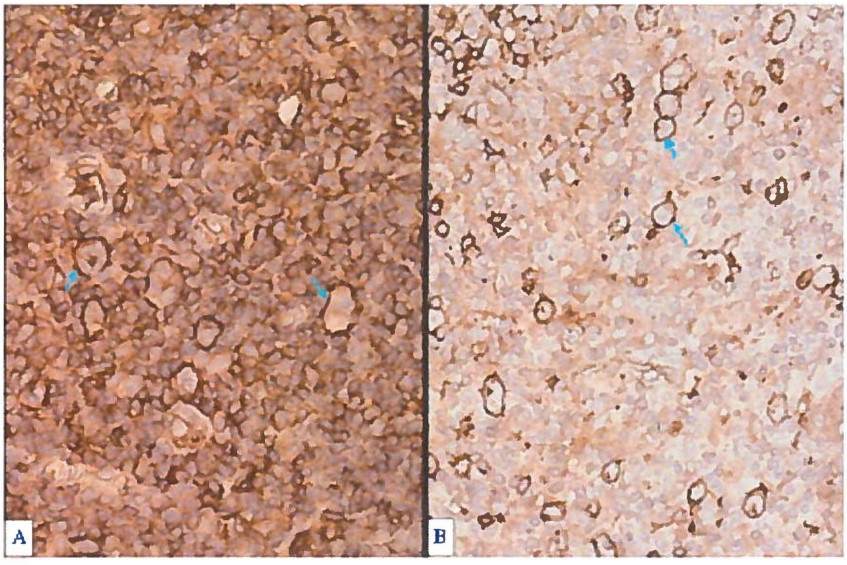

Fig 2 Immunohistochemical detection of Human Leukocyte Antigen(HLA) expression on formalin-fixed paraffin-embedded tissue sections of classical Hodgkin lymphoma(cHL). (A)HLA class I(using the HC-10 antibody) expression in cHL. All cells show immunoreactivity on the membrane. Hodgkin and Reed Stemberg(HRS) cells stand out with their stronger signal(arrow) (original magnification x40). **(B)**HLA class II(CR3/43) expression in cHL. Most HRS cells are positive(arrow), surrounded by negative inflammatory cells(original magnification ×40).

HLA class I and HLA class II expression

Expression of HLA class I heavy chains was consistent with that of 2-microglobulin and the rate of positivity was 50% (n=72). In most patients with HLA class I positive tumor cells, the HRS cells showed a higher staining intensity than the reactive background cells, especially in EBV+cases(see figure 2A). For HLA class II expression by tumor cells, 46% of patients(n=67) were positive. Usually, the HRS cells were surrounded by HLA class II negative reactive cells (see figure 2B).

In 26% of patients(n=38) there was co-expression of HLA class I and class II, whereas in 30% of patients(n=44) the tumor cells were double negative. Although there was a trend for HLA class I negative cases to also be HLA class II negative, and vice versa, this correlation was not statistically significant(Table 2).

Correlation between expression of HLA, EBV status and clinicopathologic variables

The results of HLA class I and HLA class II expression in relation to EBV status, the different histological subtypes, sex and age are summarized in Table 3. Expression of HLA class I was significantly more frequent in EBV+ than in EBV-neg cases ($P<0.001$). Histological subtypes correlated with HLA class I expression, with the highest frequency of positive expression in the MC subtype (32/42=76%) and the lowest in the NS subtype(22/75=29.3%)($P<0.001$). Using multivariate logistic regression analysis a significant effect of EBV status on HLA class I expression was observed after adjusting for histological subtype ($P<0.001$). However subtype did not remain significant when correcting for EBV status implying that EBV status explained the observed association between histological subtype and HLA class I expression. In contrast, HLA class II expression was not associated with EBV or subtype. Neither HLA class I nor

HLA class II expression was found to correlate with patients' age. In addition, our data showed that Chinese female cHL patients more frequently maintained expression of HLA class II(P=0. 01), but not expression of HLA class I.

Comparison between Chinese and Dutch cHL patients

The data from the current Chinese population were compared with data from a population based study in the Netherlands, performed by our group(12). EBV positivity and MC subtype were more common in Chinese cHL patients than in Dutch cHL patients (40% vs. 33% and 29% vs. 11%, respectively). In the Chinese cHL patients, the proportion of children was higher(age<18:22. 8% vs. 9. 2%). while that of the elderly was lower (age>60:9. 0% vs. 15. 8%). A similar association of HLA class I expression with EBV status was observed in both populations. However, expression of HLA class II highly correlated with HLA class I in the Dutch population, which was not the case in Chinese patients. Loss of HLA class II expression by HRS cells was more common in Chinese compared to Dutch patients(54% vs. 41%). This difference was independent of sex, EBV status and histological subtype, shown using a multivariate logistic regression model (P=0. 012). Table 4 shows that in Dutch patients the expression of HLA class II was strongly associated with EBV positivity (P=0. 005). Also, the NS subtype was more frequently deficient in HLA class II expression than the MC subtype (P=0. 016). Neither of these associations was observed in the Chinese cHL patients. However, only in the Chinese cHL patients an association between sex and HLA class II expression was observed (P=0. 01).

Table 2 Correlation between HLA class I and class II expression

	HLA class I Positiven=72	P‡ Negativen=73	
HLA class II			
Positive(n=67)	52. 3% (n=38)	39. 7% (n=29)	0. 115
Negative(n=78)	47. 7% (n=34)	60. 3% (n=44)	

‡ Chi square test

Table 3 HLA class I and class II expression by HRS cells in relation to EBV status, histology, sex and median age

	HLA class I			**HLA class II**		
	Positive n=72	Negative n=73	P‡	Positive n=67	Negative n=78	P‡
EBV						
Pos.	64% (n=46)	16% (n=12)	< 0. 001	45% (n=30)	36% (n=28)	0. 277
Neg.	36% (n=26)	84% (n=61)		55% (n=37)	64% (n=50)	
Histology						
NS	31% (n=22)	72% (n=53)	< 0. 001*	51% (n=34)	53% (n=41)	0. 955*
MC	44% (n=32)	14% (n=10)		27% (n=18)	31% (n=24)	
LR	3% (n=2)	0% (n=0)		1% (n=1)	1% (n=1)	
NOS	22% (n=16)	14% (n=10)		21% (n=14)	15% (n=12)	
Sex						
Male	67% (n=48)	66% (n=48)	0. 907	55% (n=37)	75% (n=59)	0. 01
Female	33 % (n=24)	34% (n=25)		45% (n=30)	25% (n=19)	
median age (range)	33(4~74)	26(6~61)	0. 246	28(4~74)	28(4~74)	0. 666†

‡ Chi square test, * Fisher's exact test, NOS histology not included, † Mann Whitney U test, NS indicates nodular sclerosis; MC, mixed cellularity; LR, lymphocyte rich; NOS, not otherwise specified.

Table 4 Comparison of the association of HLA class II expression with EBV status and histological subtypes between Dutch* and Chinese cHL patients

	Dutch patients*			Chinese patients		
	HLA class II		P‡	HLA class II		P‡
	Positive (n=171)	Negative (n=121)		Positive (n=67)	Negative (n=78)	
EBV						
Positive	39. 8% (n=68)	24. 0% (n=29)	0. 005	44. 8% (n=30)	35. 9% (n=28)	0. 277
negative	61. 2% (n=103)	76. 0% (n=92)		55. 2% (n=37)	64. 1% (n=50)	
Histology$						
NS	84. 1% (n=143)	87. 3% (n=103)	0. 016†	64. 1% (n=34)	62. 1% (n=41)	0. 796†
MC	14. 7% (n=25)	5. 1% (n=6)		34. 0% (n=18)	36. 4% (n=24)	
LR	0. 6% (n=1)	3. 4% (n=4)		1. 9% (n=1)	1. 5% (n=1)	
LD	0. 6% (n=1)	4. 2% (n=5)		0	0	
Sex						
Male	57. 9% (n=99)	58. 7% (n=71)	0. 89	55. 2% (n=37)	75. 6% (n=59)	0. 01
Female	42. 1% (n=72)	41. 3% (n=50)		44. 8% (n=30)	24. 4% (n=19)	
Median age (range)	34(8~88)	32(8~88)	0. 85	28(4~74)	28(4~74)	0. 666#

‡Chi square test, * Data have been published(Diepstra A et al(12), † Comparison was made between NS and MC subtype only, # Mann Whitney U test, $ Classical Hodgkin lymphoma not otherwise specified excluded, NS indicates nodular sclerosis; MC, mixed cellularity; LR, lymphocyte rich; LD, lymphocyte depleted

Discussion

This study involved the largest group of northern Chinese cHL patients evaluated for expression of HLA class I and HLA class II by HRS cells. HLA class I expression was strongly associated with EBV-positivity, similar to the Caucasian populations. However, an association between HLA class II expression and EBV status was not apparent, in contrast to the data from Western Europe.

The EBV association with cHL has been investigated by several research groups within different geographic locales and ethnicities, showing a common association between EBV-associated cHL, male sex and MC subtype (3, 5). These associations were also present in this northern Chinese patient population.

Although the pathogenesis of cHL is still not clearly established, immune escape mechanisms seem to play an important role, especially in cases that are EBV-associated. Downregulation of HLA on the surface of tumor cells has been observed in various types of human malignancies, including cHL(18). Our present data demonstrate that deficiency in membranous expression of HLA antigen by tumor cells is quite common in EBV-neg cHL, since 49. 7% and 46. 2% cases expressed HLA class I or class II respectively and only 26. 2% cases expressed both. Lack of HLA expression is generally thought to be advantageous for the persistence of tumor cells by escaping T cell mediated immunosurveillance.

An intriguing finding is that HLA class I expression is usually retained in EBV+ cases, although HLA class I-restricted CTL responses in general play a major role in eliminating virus infection. This correlation is consistent with the results of four previous studies performed on Caucasian cHL populations from the Netherlands and the United Kingdom(13, 14, 16). Our data also show a correlation between HLA class I expression and MC subtype, which can be attributed to the

close relationship of EBV involvement with MC subtype. It is apparent that not only the quantity, but also the quality of HLA molecules determines the ability to mount an immune response. The composition of HLA alleles determines the peptide binding ability and the subsequent immune response. A recent report on a detailed investigation within the HLA region lends proof to this concept, as patients with EBV-associated cHL more frequently carry HLA-A*01 in comparison to EBV-negative cHL patients who usually carry HLA-A*02 instead (11, 15). It should be noted that persistence of HLA class I expression can be advantageous for the tumor cells to survive Natural Killer cell-mediated lysis(19).

HLA class II-restricted CD4 T cell immune responses are largely, but not exclusively restricted to exogenous antigens and it has become evident that these responses are essential for an efficient eradication of EBV infection(10). Our group previously reported a significant association of HLA class II expression with EBV status in 292 Dutch cHL patients. In contrast, we did not find this association in the Chinese population in the current study, although we could see a slight non-significant tendency to maintain the expression of HLA class II in EBV-associated cHL.

In addition, the previously reported correlation between the expression of HLA class I and class II(12) was absent in the present study. A major difference between the Dutch and Chinese population is the percentage of MC subtype patients with 10. 6% and 28. 9% of patients respectively. Strikingly, the expression of HLA class II in MC subtype patients was very different between the two populations and was much more frequently downregulated in the Chinese patients. Since we used the same antibody, staining protocols and scoring criteria for evaluating HLA class II expression in both populations, the differential expression likely reflects a biological difference between the two different populations which presumably relates to HLA-based genetic heterogeneity. The

HLA system is extremely polymorphic and allelic frequencies vary dramatically between racial groups. A certain HLA allele that is relatively uncommon in one population can be highly prevalent and associated with a specific disease in another. In the Chinese population one or more prevalent HLA class II allele(s) might present immunodominant EBV antigenic peptides to the immune system, thereby exerting selection pressure to downregulate this molecule. Thus, yet-to-be-defined ethnic-specific HLA alleles are likely to affect the strength of association between HLA class II expression and the strongly EBV associated MC subtype. It should be noted that Oudejans et al (16) also studied the expression of HLA class II in Dutch cHL patients, but did not find the same association. However, they used another antibody, recognizing HLA-DR only, the sample size of their study was much smaller(n=63) and they used a different set of scoring criteria(16).

An additional association found in the Chinese population was a strong correlation between downregulation of HLA class II with male sex. Since male sex is associated with EBV+ tumor cell status in general, this might partially explain the lack of association of HLA class II with EBV status in the Chinese patients. However, there is no obvious explanation for the association of HLA class II expression with sex. Interestingly, lack of HLA class II expression is related to inferior failure free survival in the Dutch population(12). An important consequence of the differences in HLA class II expression between the Chinese and Dutch patients might be that this adverse prognostic impact of HLA class II absence is not necessarily present in other populations.

In conclusion, our data demonstrate that in northern Chinese patients, EBV + cHL tumor cells more frequently retain HLA class I expression, similar to Caucasian populations. However, the association of HLA class II expression with positive EBV status, as observed in Caucasians, is not present in the northern

Chinese population. Investigation at the molecular level is needed to further explore the role of anti-tumor immune responses in the pathogenesis of cHL. Differences in ethnic background should be taken into account and might explain discrepancies in incidence pattern, EBV association and other aspects in different populations.

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Chapter 4

HLA-A*02:07 is a susceptibility allele for EBV positive classical Hodgkin Lymphoma in China

Xin Huang[1, 2], Bouke Hepkema[3], Ilja Nolte[4], Kushi Kushekhar[1], Theo Jongsma[3], Sibrand Poppema[1], Zifen Gao[2], Arjan Diepstra[1] & Anke van den Berg[1]

[1]Department of Pathology and Medical Biology, [3]Department of Laboratory Medicine, [4]Unit of Genetic Epidemiology and Bioinformatics, Department of Epidemiology, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands. [2]Department of Pathology, Health Science Center, Peking University, Beijing, China.

Submitted

Abstract

Background: HLA-A2 protects against Epstein-Barr virus (EBV)-associated classical Hodgkin Lymphoma(cHL) in the Caucasian population, whereas HLA-A1 is a risk allele. In the Chinese population HLA-A1 is uncommon and the prevalence of HLA-A2 subtypes is quite different from that in Caucasians. In this study, we investigated the association between HLA-A*02 and specific HLA-A*02 subtypes with EBV+cHL in the Chinese population.

Methodology/ Principal Findings: Formalin-fixed, paraffin embedded tissue blocks of 161 cHL patients and 119 controls with benign lymphadenopathy from the northern part of China were retrieved. EBV status was determined by in situ hybridization for EBERs. DNA was extracted from the tissue blocks and HLA-A2 quantitative(q)PCR was performed to discriminate between negative and positive (including both homozygous or heterozygous) samples. HLA-A2 positive samples were subtyped to discriminate between "common and well-documented" HLA-A2 subtypes by sequence-based typing. 67(43%) of the cHL patients were EBV+. There were no significant differences in percentages of HLA-A2 positivity between cHL and controls(65% vs 66%) and between EBV+and EBV- cHL patients(70% vs 61%). The frequency distribution of HLA-A2 subtypes was similar for patients and controls. The HLA-A*02:07 allele frequency was significantly higher in the EBV+cHL(36%) as compared to the EBV-(7%) and the control group(19%). The frequency of all other HLA-A2 subtypes combined was clearly decreased in EBV+patients(30%) compared to EBV- patients(51%) and controls(45%).

Conclusion: HLA-A*02:07 is a predisposing allele for EBV+cHL, while all other HLA-A*02 subtypes are protective in the Chinese population. Our findings demonstrate the influence of HLA allele differences in ethnic groups on epidemiological heterogeneity of cHL.

Introduction

Classical Hodgkin lymphoma (cHL) has a complex multi-factorial etiology with both genetic and environmental factors contributing significantly to its development(1, 2). Epstein-Barr virus (EBV), a ubiquitous human gamma-herpes virus that infects over 90% of the population worldwide (3), has been consistently linked to the pathogenesis of a subset of cHL(1). Despite its strong oncogenic potential, only a minority of EBV-infected individuals develop EBV + cHL (3), probably because of effective anti-viral immune responses.

The incidence of EBV+cHL is strikingly increased in immunocompromised patients(4) indicating the importance of an effective immune system in controlling the EBV+cell population. The human leukocyte antigen(HLA) is a crucial element of the human immune system and HLA class I restricted, CD8+cytotoxic T-cell (CTL) responses are known to play a pivotal role in the control of EBV infection(5). In EBV+cHL, expression of viral genes by the tumor cells is restricted to the two latent membrane antigens(LMP1 and LMP2) and EBV nuclear antigen 1(EBNA1), which is a so-called latency type II infection(6). Induction of LMP/EBNA1-specific immune responses has been successful in the context of appropriate HLA molecules(7-9).

Genetic association between HLA and cHL has been widely investigated in the Caucasian population and HLA-A2 has been consistently reported as a protective allele for developing EBV + cHL, while HLA-A1 is considered a risk allele(10, 11). HLA-A2 has a high prevalence worldwide(12) and is one of the most diverse allele families of the HLA-A locus consisting of over 300 allelic variants (IMGT/HLA database v 3. 3. 0). These alleles show a marked difference in the capacity of peptide binding and presentation (13). For example, presentation of EBV derived peptides by the HLA-A*02: 01 allele induces stronger immune responses as compared to other HLA-A*02 sub alleles, including HLA-A*02:07

(14). The distribution of these HLA-A*02 allelic variants differs widely by geography and ethnicity(12). In the Caucasian population, > 90% of the HLA-A2 positive individuals carry the HLA-A*02:01 allele (12), whereas in the Chinese population there are multiple common and well-documented (CWD) allelic variants, including HLA-A*02:01, A*02:03, A*02:06, A*02:07 and A*02: 10 (15). Based on these marked differences, it can be postulated that inter-racial variations influence the host immune response and this may lead to different HLA-A2 disease associations in different populations.

Consistent with Caucasian populations, in the vast majority of Chinese EBV+cHL patients the tumor cells express HLA class I at the cell surface(16). In this study, we determined the HLA-A*02 phenotype frequency in the same group of Chinese cHL patients as well as in controls from the same geographic region. The CWD HLA-A*02 allelic variants were determined by sequencing-based typing(SBT).

Materials and Methods

Study Population

Formalin-fixed paraffin-embedded(FFPE) tissue blocks of lymph node biopsies from 185 cHL patients obtained from 5 hospitals in northern China (Dept. of Pathology, Health Science Center, Peking University; Dept. of Pathology, First Hospital of Jilin University; Dept. of Pathology, Shougang Hospital, Peking University; Dept. of Pathology, Beijing Air Army General Hospital; Zhanye Regional Hospital, Gansu Province) were used for this study. Results of the hematoxylin & eosin(H&E) staining, reclassification according to the WHO classification and EBER in situ hybridization have been published previously(16). DNA was extracted from the tissue blocks. Of the 121 cHL patients for whom the HLA-A*02 DNA quality was sufficient for HLA-A*02 qPCR, 44(36%) patients were EBV+as previously determined by ISH for EBERs.

To minimize genetic variations we included a control group of 124 reactive lymph

nodes with lymphoid reactive hyperplasia(LRH) or Kikuchi Disease obtained during the same period from the Dept. of Pathology, Health Science Center, Peking University as a control group.

FFPE tissue blocks of 21 cHL patients with known HLA genotype were retrieved from the University Medical Center Groningen(UMCG), the Netherlands. These samples were used to optimize and validate the HLA-A*02 specific quantitative(q)PCR. These 21 cases consisted of 6 A*02-homozygotes, 7 A*02-heterozygotes and 8 non-A*02 genotypes. All procedures were carried out according to UMCG METc guidelines.

DNA isolation

2~3 10μm tissue sections were incubated overnight at 55℃ in 242. 5μL of PK1 lysis buffer(10mM Tris pH 8. 0, 50mM KCl, 2. 5mM MgCl2, 0. 45% NP40, 0. 45% Tween 20, 0. 01% gelatin) with 7. 5μL Proteinase K (20mg/ml, Invitrogen). Proteinase K was heat inactivated for 5 min at 100℃. The supernatant containing the DNA was carefully transferred to a new tube. DNA was quantified by nanodrop-1000 spectrophotometer and diluted to a concentration of 5 and 10ng/μL for the subsequent PCR analysis.

HLA-A*02 quantitative (q) PCR

qPCR assays were carried out on an ABI PRISM 7900HT(Applied Biosystems) with SYBR-green in a 384-well microtiter plate using primers specific for HLA-A2 and as a control quality and quantity we used a primer set for the PTP4A1 gene. All reactions were performed in triplicate in a final reaction volume of 20μl consisting of 10μl SYBR? Green PCR master mix(Applied Biosystems), 2μl(3mM) of each primer and 5μl (5ng/μl) DNA. Each sample was analyzed in triplicate, starting with a 2-min AmpErase UNG activation step at 50℃ and a 10-min hot start at 95℃, followed by 45 cycles of denaturation at 95℃ for 15s and combined annealing/extension at 60℃

for 1min. A melting curve was generated(95℃ for 15s, 60℃ for 15s, and 95℃ for 15s) to verify specificity of the PCR products. Cycle threshold (Ct) values were determined by using the default baseline setting of 3 to 15 cycles. All data were analyzed by using ABI PRISM 7900 HT Sequence Detector Systems software version 2. 3(Applied Biosystems). Samples with poor DNA quality were excluded based on lack of a specific peak in the melting curve or a Ct value higher than 36 for PTP4A1(5 controls and 24 patients were excluded). HLA-A*02 Ct values were normalized against the Ct value for PTP4A1, resulting in a delta Ct. Relative abundance of HLA-A*02 was calculated by using the formula 2 ΔCt. Based on the 21 previously HLA-A typed samples, cut-off levels for HLA-A*02 positivity were set at a 2-ΔCt value of 0. 1. A 2-ΔCt value of 0. 05 or less was considered to indicate absence of HLA-A*02.

HLA-A*02 subtypes

Subtyping of HLA-A2 positive cases was performed by amplification and sequence analysis of exon 2 and exon 3 regions that contain a number of SNPs that discriminate between the common and well-documented (CWD) allelic variants in the Chinese population(Table1, Figure 1). DNA samples were amplified in a volume of 25μl containing 5μl(10ng/μl) DNA, 5μl(2mM) of each primer, 1. 25μl dNTPs(Invitrogen), 2. 5μl 10X reaction buffer(Invitrogen), 5. 85μl H2O and 0. 4μl Taq Polymerase (Invitrogen). Amplification was performed in a GeneAmp PCR System 9700 thermocycler(Applied Biosystems) with an initial 3-min hold at 95℃ and 40 cycles of 15s at 95℃, 30s at 65℃, and 1 min at 68℃, followed by a final 10min extension step at 68℃. PCR products were visualized on a 1% agarose gel and purified by Exo-SAP treatment(USB products, Affymetyrics, Cleveland, Ohio USA). PCR products were sequenced using the same primers as the ones used for amplification. Due to the longer amplicon size of the exon 2 PCR products a number of samples failed for the analysis of the SNPs in this region leading to a less effective

discrimination between the CWD HLA-A*02 subtypes in these samples. This led to ambiguities for some alleles. Using the primer set as indicated above we were not able to discriminate between HLA-A*02:01 and HLA-A*02:03, leading to a HLA-A*02:01/ 02:03 ambiguity. Sequence data were analyzed using Seqman software (DNA Star, Madison, WI). Percentages were based on the frequency within the HLA-A2 positive groups. We counted both HLA-A2 alleles separately for heterozygous individuals.

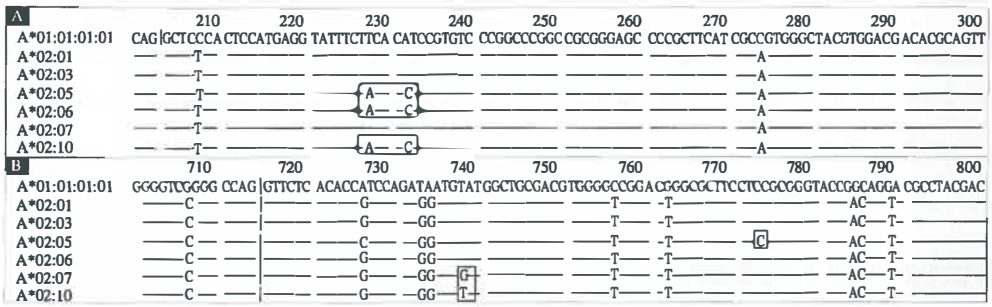

Figure 1 Alignment of the HLA-A*02 gene fragments that are amplified and sequenced to discriminate between common and well defined HLA-A*02 allele. The four SNPs that are able to discriminate between the major HLA-A*02 subtypes(IMGT/HLA database, http://www. ebi. ac. uk/imgt/hla/) are indicated by boxes. **(A)** Two SNPs located at position 228 and 231 are specific for HLA-A*02:05, A*02:06 and A*02:10. **(B)**Another SNP located at position 773 is specific for HLA-A*02:05 only and one SNP at position 739 can differentiate both HLA-A*02: 07 and A*02: 10 from others. Numbers above the sequences indicate nucleotide positions. Nucleotide sequences were aligned to the HLA-A*01:01 allele. Dashes indicate identity with the reference sequence. The intron1-exon 2 **(A)**and exon2-exon3 **(B)**junctions are denoted by vertical bars.

Table1 Primer sequences for allelic discrimination

Allele specificity	Primer sequence*(5′ — 3′)	Location	Product size
HLA-A*02	F:GAGCCCCGCTTCATCGCA	Exon 2	150bp
	R:CCCGTCCCAATACTCCGGA	Exon 2	
PTP4A1	F:GCACAGCACGACCTCTATGC	Exon 2	142bp
	R:CCAGGTCAGAACTCTTGTAAAATGC	Exon 2	
HLA-A*02 exon 2	F:CTCTGTGGGGAGAAGCAAC	Intron 1	191bp
	R:GTCGTCCACGTAGCCCACT	Exon 2	
HLA-A*02 exon 3	F:GCGGGGCTCGGGGGACC	Intron 2	131bp
	R:GCCGTCGTAGGCGTACTGG	Exon 3	

* F, forward primer; R, reverse primer. Nucleotides in bold indicate the positions of the SNPs specific for the CWD HLA-A*02 alleles.

Statistical Analysis

Significant differences in HLA-A*02 positivity and in phenotype frequency of HLA-A*02 subtypes between the total cHL group and the control subjects as well as those between EBV+and EBV- cHL patients were assessed by chi-square tests. All statistical analyses were performed in Microsoft Excel 2003.

Results

Validation of the qPCR

Analysis of the 21 cHL samples with a previously typed HLA-A genotype revealed a clear separation between individuals lacking HLA-A2 alleles and individuals heterozygous or homozygous for the HLA-A2 allele (figure 2). The relative expression values varied between 0. 002 and 0. 027 for negative patients and between 0. 63 and 4. 26 for positive patients.

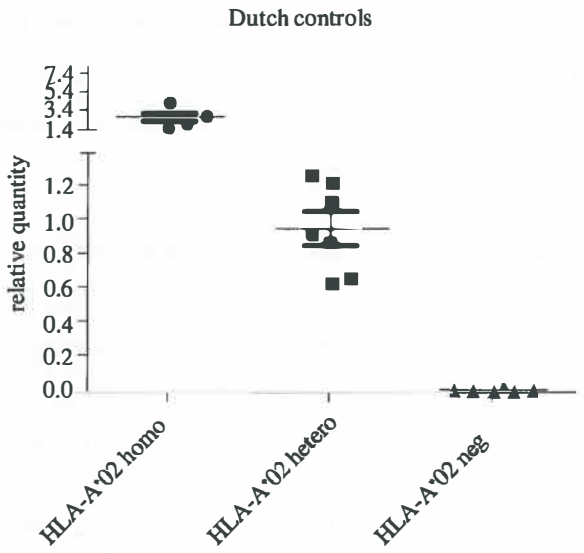

Figure 2 Validation of the HLA-A*02 specific primer set on individuals with known HLA genotypes. A clear difference can be observed between samples possessing at least one HLA-A2 allele and those without HLA-A2 alleles.

HLA-A2 carrier frequency

24 cHL patients and 5 controls were excluded due to bad DNA quality. HLA-A*02 carrier frequency of the remaining 161 cHL patients and 119 controls revealed no significant difference between the total cHL patient group(104 out of 161, 65%) and the control group(79 out of 119, 66%). In contrast to the strong significant association observed in the Western population, no difference was observed between the HLA-A2 frequency in EBV+(47 out of 67, 70%) and EBV-(57 out of 94, 61%) chinese cHL patients(Table 2).

HLA-A2 subtype frequencies

HLA-A*02 subtyping failed in 5 cHL patients and 3 controls due to inefficient amplification of both exons and/or poor sequencing results. For part of the patients subtyping was based only on the exon 3 sequence analyses, which did

not allow discrimination between all CWD alleles. SBT results of the 101 cHL patients and 76 controls are shown in Table 3. Eight patients and seven controls showed a heterozygous pattern for two HLA-A2 alleles, whereas in all other cases only one HLA-A2 allele was observed(Table 3).

Table 2 Phenotype frequencies of HLA-A*02 in Chinese patients with cHL and controls

	Controls	All patients	EBV+cHL	EBV-cHL
	N=119 (%)	N=161 (%)	N= 67 (%)	N= 94 (%)
HLA-A*02-pos	79 (66. 4)	104 (64. 6)	47 (70. 1)	57 (60. 6)
HLA-A*02-neg	40 (33. 6)	57 (33. 4)	20 (29. 9)	37 (39. 4)

Table 3 CWD HLA-A2 alleles in HLA-A2 positive controls and cHL patients

	76 controls	99 cHL patients	44 EBV+cHL	55 EBV-cHL
HLA-A*02 allelesa	n= 83	n= 107	n= 50	n= 57
A*02:01/02:03	27 (33. 8%)	31 (30. 4%)	9 (19. 1%)	22 (40. 0%)
A*02:01/02:03/02:06	17 (21. 3%)	16 (15. 7%)	7 (14. 9%)	9 (16. 4%)
A*02:01/02:03/02:05/02:06	2 (2. 5%)	0 (0. 0%)	0 (0. 0%)	0 (0. 0%)
A*02:05	1 (1. 3%)	1 (1. 0%)	0 (0. 0%)	1 (1. 8%)
A*02:06	11 (13. 8%)	25 (24. 5%)	9 (19. 1%)	16 (29. 1%)
A*02:05/02:06/02:10	1 (1. 3%)	0 (0. 0%)	0 (0. 0%)	0 (0. 0%)
A*02:07	23 (28. 8%)	31 (30. 4%)	24 (51. 1%)	7 (12. 7%)
A*02:10	1 (1. 3%)	3 (2. 9%)	1 (2. 1%)	2 (3. 6%)
failure	3 (3. 6%)	5 (4. 7%)	3 (6. 0%)	2 (3. 5%)

[a] Five patients and one control were heterozygous for HLA-A*02:03 and HLA-A*02:07, two patients and one control were heterozygous for HLA-A*02:06 and HLA-A*02:07, one patient was heterozygous for HLA-A*02:06 and HLA-A*02:10, one control was heterozygous for HLA-A*02:05 and HLA-A*02:07, one control for HLA-A*02:01/02:03/02:06 and HLA-A*02:07 and three controls were heterozygous for HLA-A*02:01/02:03 and HLA-A*02:06.

The HLA-A*02:01/02:03 ambiguity was most common in both controls and patients. The second most common allele in both controls and cHL patients was HLA-A*02:07 and HLA-A*02:06 was the third most common allele. HLA-A*02:05

and A*02:10 were rare in patients and controls.

In contrast to the similar distribution of HLA-A2 subtypes in controls and the total cHL patient group, the HLA-A2 subtype frequencies varied greatly between EBV+and EBV- cHL patients. The HLA-A*02:07 allele frequency was very high in EBV+cHL patients(24 out of 50, 48%) and low in EBV- cHL patients(7 out of 57, 12%). All other HLA-A * 02 subtypes were less common in EBV + cHL as compared to the EBV- cHL patient group.

Table 4 HLA-A*0207 carrier frequency in Chinese controls and cHL patients

	Controls		All patients		EBV+cHL		EBV-cHL	
HLA type	N	%	n	%	n	%	n	%
A*02:07	23	19. 8%	31	19. 9%	24	37. 5%	7	7. 6%
A*02 (non 02:07)	53	45. 7%	68	43. 6%	20	31. 3%	48	52. 2%
A*02 negative	40	34. 5%	57	36. 5%	20	31. 3%	37	40. 2%
failure	3		5		3		2	

HLA-A* 02:07 risk allele analysis

Analysis of the frequency distribution of HLA-A * 02 negative, the HLA-A * 02: 07 positive and the A*02(non 02:07) positive cases in controls, total, EBV+and EBV- cHL patient groups revealed significant differences(Table 4). The odds ratio(OR) for HLA-A*02:07 was strongly increased in EBV+cHL as compared to EBV- cHL (OR= 6. 34, CI 2. 33-17. 28) and controls(OR= 2. 09, CI 0. 95-4. 57). The HLA-A* 02(non 02:07) OR was strongly reduced in the EBV+cHL subgroup as compared to EBV- cHL(OR= 0. 77, CI 0. 36-1.6mm4) and controls(OR= 0. 75, CI 0. 36-1. 59). In the EBV- cHL group the OR for HLA-A*02:07 was reduced(OR= 0. 33, CI 0. 13-0. 86) but it was not changed for HLA-A*02(non 02:07) in comparison to normal controls(Table 5).

Table 5 HLA-A*02:07 allele frequency in controls and cHL patients stratified by EBV status

HLA-A2 suballele	Controls vs EBV+ OR (95% CI)[a]	p-valueb	Controls vs EBV- OR (95% CI)[a]	p- valueb	EBV+ vs EBV- OR (95% CI)a	p-value[b]
A*02:07	2.09 (0.95-4.6)	0.028	0.33 (0.13-0.86)	0.045	6.34 (2.3-17)	2×10^{-5}
A*02 (non 02:07)	0.75 (0.36-1.6)		0.98 (0.54-1.8)		0.77 (0.36-1.6)	

[a] HLA-A02 negative is reference group,[b] Significance of the 2df chi-square test comparing A*02:07,A*02:XXand A*02 negative frequencies between groups

Discussion

HLA-A2 has been identified as a protective allele and HLA-A1 as a risk allele for the development of EBV+cHL in the Western population(10, 11). In this study, we found no difference in the HLA-A2 allele carrier frequency between EBV + cHL patients and controls from Northern China. A further discrimination between the CWD HLA-A2 subtypes in the Chinese population revealed that HLA-A * 02: 07 allele was a risk allele for EBV+cHL, whereas all other HLA-A*02(non 02:07) were protective. The HLA-A1 frequency was not tested because this allele is uncommon in the Chinese population.

HLA-A*02:07 is almost exclusively present in the eastern Asian population and is the second most common allele just after the HLA-A*02:01 allele(13). In the southern Chinese population the HLA-A*02:07 allele frequency was reported to be even higher than the HLA-A*02:01 frequency(15). The proportion of HLA-A*02:07 positive individuals was slightly higher than the frequency reported in the northern Chinese population(15).

Difference between the HLA-A*02:01 allele and the HLA-A*02:07 allele at the protein level is only one amino acid(Y99 to C) and this variation is not present in the HLA-A*01 allele. The question arises if this single amino acid difference can indeed make a difference between a risk and a protective allele. An explanation for the observed differences might be found in differential specificity of binding and presentation of antigenic peptides(13). Indeed, HLA-A*02:01 has been shown to be more effective in binding and presentation of EBV-derived peptides in comparison to other HLA-A*02 subtypes including HLA-A*02:07 (17-19). Interestingly, the HLA-A*02:07 allele is also associated with an increased risk of undifferentiated nasopharyngeal carcinoma (UNPC) in the southern Chinese population(20). UNPC is an EBV-associated malignancy and has an identical latency type of infection as compared to cHL with an expression pattern that is

restricted to LMP1, LMP2 and EBNA1(6, 21). In the Caucasian population HLA-A *02:01 was associated with a decreased risk for UNPC(22). These findings are similar to our findings in cHL and support a role for the effectiveness of the immuneresponse against latency type II proteins in the pathogenesis of both diseases.

In conclusion, our study reported for the first time that the HLA-A*02:07 allele has a strong predisposing effect for EBV+ cHL in the Chinese population, while other HLA-A*02(non 02:07) alleles were protective. Discrimination between HLA-A*02 subtypes was of central importance for investigating the genetic association of HLA-A*02 with EBV+ cHL. Differences in Chinese and Caucasian populations provides a unique opportunity for further understanding the influence of genetic heterogeneity in HLA and its role in the pathogenesis of EBV+ cHL.

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Chapter 5

Multiple HLA class I and II associations in classical Hodgkin lymphoma and EBV status defined subgroups

Xin Huang[1, 2], Kushi Kushekhar[1], Ilja Nolte[3], Wierd Kooistra[1], Lydia Visser[1], Ilby Bouwman[5], Niels Kouprie[5], Rianne Veenstra[1], Gustaaf van Imhoff[4], Sibrand Poppema[1], Arjan Diepstra[1], Bouke Hepkema[5] and Anke van den Berg[1*]

[1]Department of Pathology and Medical Biology, [3]Department of Laboratory Medicine, [4]Unit of Genetic Epidemiology and Bioinformatics, Department of Epidemiology, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands. [2]Department of Pathology, Health Science Center, Peking University, Beijing, China.

Submitted

Abstract

Background: The pathogenesis of classical Hodgkin lymphoma (cHL) involves environmental and genetic factors.

Methodology/Principal Findings: To explore the role of the human leukocyte antigen(HLA) genes, we performed a case-control genotyping study in 338 Dutch cHL patients and more than 5,000 controls using a PCR-based sequence-specific oligonucleotide probe(SSOP) hybridization approach. HLA-DR7 was significantly decreased and HLA-B51 significantly increased in the cHL patient population as compared to the controls. Three class II associations were observed in the EBV- cHL population with an increase of HLA-DR15(2) and a decrease of HLA-DR4 and HLA-DR7. Allele frequencies of HLA-A1, HLA-B37 and HLA-DR10 were significantly increased in the EBV+cHL population and these alleles are in strong linkage disequilibrium forming a common haplotype in caucasians. The allele frequency of HLA-DR11 (5) was significantly decreased in the EBV + cHL population. SSOP analysis revealed significant differences between EBV + and EBV- cHL patients for 19 probes that discriminate between HLA-A*01 and HLA-A*02.

Conclusion/Significance: In conclusion, the HLA-A1 and HLA-A2 antigens and not a specific single nucleotide variant shared by multiple alleles are responsible for the association with EBV+cHL. Several new protective or predisposing HLA class I and II associations for the EBV+, the EBV- and the entire cHL population were identified.

Introduction

Classical Hodgkin lymphoma(cHL) is a typical multi-factorial disease with both environmental and genetic factors acting together to cause disease (1, 2). Epidemiological studies reporting familial clustering of cHL(3) and racial variation in the incidence of cHL(4) gave substantial support for an inherited risk to cHL. Genetic associations with specific human Leukocyte Antigen(HLA) alleles have been reported in both sporadic and familial cHL(5, 6).

Epstein Barr virus(EBV) is a well-established causal factor in a subset of cHL patients (7). The expression pattern of EBV genes in Hodgkin Reed-Sternberg(HRS) cells is restricted to the two latent membrane proteins(LMP1 and LMP2) and the EBV nuclear antigen 1 (EBNA1) (7). Despite the lack of immunodominant EBV proteins, LMP and EBNA1-specific T cell responses can be efficiently induced in the context of specific HLA class I or class II molecules (8-10). The extreme diversity of HLA genes influences both the affinity and specificity of antigenic peptide binding and is responsible for variations in host anti-viral immune defenses. Genetic variation in host anti-viral immune responses related to HLA polymorphisms might be an important contributor to the development of virally induced malignancies.

Initial HLA association studies in cHL were performed without taking EBV status into account and associations of HLA-A1, HLA-B5, HLA-B8 and HLA-B18 with cHL have been described, although the degree of reproducibility has been low(6, 11-13). More recently, we focused on the EBV+cHL subgroup by genetic screening the entire HLA region and subsequent finescreening and found a strong association of specific HLA-A alleles with susceptibility to EBV+cHL in Dutch and English patients5, (14). The HLA-A1 was associated with an increased risk for EBV+cHL, whereas the HLA-A2 was associated with a decreased risk for EBV+cHL(15). This association was confirmed in 934 Scandinavian and English

cHL patients in a recent study by Hjalgrim et al(16).

In the present study, we performed an extensive screen of the HLA class I and II genes to investigate possible associations of HLA alleles in the total, the EBV+ , and the EBV- cHL (sub) populations. Furthermore, we intended to establish whether specific single nucleotide polymorphism(SNP) positions in the HLA genes that might be shared by multiple HLA alleles are responsible for the observed genetic associations rather than specific HLA alleles.

Materials and methods

Patients and controls

183 Dutch cHL patients who participated in the previous genotyping study 5 were included in the present study. 155 additional patients diagnosed and/or treated between 2000 and 2010 at the University Medical Center Groningen in the Netherlands were also included. Data on gender, age, and histopathological diagnosis was available for all these patients. Classification according to the WHO was performed consistently with the previous study and cHL cases that could not be unequivocally subtyped (usually because of little tissue) were classified as cHL not otherwise specified(Table 1). The presence of EBV in tumor cells was detected in formalin fixed paraffin embedded tissue sections by in-situ hybridization(ISH) with a fluorescein-conjugated PNA probe specific for the EBV-encoded EBER RNAs(DAKO, Glostrup, Denmark). All participants gave written informed consent. The protocol was approved by the medical ethics board of the University medical Center, Groningen. The control group consisted of blood bank donors from the same geographical region typed for HLA-A(n=7, 099), HLA-B (n=7, 283) and HLA-DR(n=5, 922) by serological methods and in case of unclear results or apparent class II homozygosity additionally by DNA based methods.

HLA genotyping

Blood samples of cHL patients were collected and genomic DNA was extracted from the peripheral blood mononuclear cell pellets using standard laboratory protocols. The HLA genotype was analyzed at medium resolution by a polymerase chain reaction-based sequence-specific oligonucleotide probe hybridization (PCR-SSOP) approach using commercial kits (Gen-Probe, San Diego, CA) and Luminex xMAP technology (Luminex Corp. , Austin, TX). The assays were performed according to the manufacturer's instruction in a European Federation for Immunogenetics accredited laboratory. Briefly, biotin-labeled amplification products were generated for exon 2 (for HLA-DRB, HLA-DPB1) and exon 3(for HLA-A, HLA-B, HLA-Cw and HLA-DQB1) of the HLA genes, followed by a hybridization reaction with a series of SSOPs. Of the 401 PCR-SSOP probes 43 were either positive or negative for all samples and were therefore excluded from the analyses. Of the 358 included probes, 63 were for the HLA-A locus, 83 for the HLA-B locus, 53 for the HLA-C locus, 72 for the HLA-DR locus, 41 for the HLA-DQB1 locus and 46 for the HLA-DPB1 locus. Each probe covered 1 to 3 SNP positions. The HLA alleles were defined by specific hybridization patterns of multiple probes. HLA genotype was ascertained according to the manufacturers instructions using the manufacturer's software and additionally by the SCORE software (18), enabling the exclusion of probes with borderline hybridization signals. The presumed antigen or T cell receptor binding function of the SNPs were identified according to Bjorkman and Parham (18).

HLA allele frequencies of the cHL patients were deducted from the PCR-SSOP genotyping data based on the Nomenclature for factors of the HLA system (http://hla. alleles. org/nomenclature/naming. html). Split antigens were used as indicated in the tables. In case of ambiguous results, the allele combination only

consisting of common and well documented alleles (19) were used. For HLA-DPB1 one ambiguity remained(DPB1*04:02/105:01) which was analyzed as one group of alleles.

Statistical analysis

For each individual PCR-SSOP association with EBV status was tested by logistic regression in PLINK v1. 07(20)(http://pngu. mgh. harvard. edu/purcell/plink/) with EBV status as a dependent variable, SSOP as an independent variable, and age and histopathological diagnosis of cHL as confounding covariates.

Allele frequencies of HLA-A, HLA-B and HLA-DR of the total cHL group, the EBV+subgroup and the EBV- subgroup were compared with allele frequencies of the controls and significant differences were assessed by Chi-square tests. Allele frequencies for HLA-C, HLA-DP and HLA-DQ of the controls were not available. Significant differences in allele frequencies between EBV+and EBV- cHL patients were also assessed by Chi-square tests. Alleles with a frequency< 1% in our population were excluded for all these analyses.

For each phenotype and PCR-SSOP a test was performed, hence a correction for multiple testing was required. Because linkage disequilibrium exists between the PCR-SSOPs and between the HLA phenotypes, and because the HLA-phenotypes are derived from the PCR-SSOPs, a Bonferroni test for 401 PCR-SSOPs and 74 phenotypes would be too conservative. Therefore, we considered p-values smaller than 0. 001 to be significant at a level of 5% and p-values <0. 003 to be suggestive for association.

Results

Clinicopathological characteristics

Characteristics of the patient population in terms of age, gender and

histopathological subtype in relation to EBV status is summarized in Table 1. EBV was present in the tumor cells in 78(25%) of the 311 cHL patients(for 27 patients EBV status was unknown). In the total group, median age at diagnosis was 35 years(range 13 to 81) and the percentage of males was 52%. 43 of 338 cHL patients could not be subtyped and were designated cHL NOS. In the remaining patients, nodular sclerosis (NS) was the most common subtype accounting for 87%. Mixed cellularity (MC) and lymphocyte rich (LR) subtypes were less common with frequencies of 10% and 3%, respectively. The lymphocyte depletion (LD) subtype was absent in this patient group. These characteristics are largely consistent with those published for Caucasian cHL populations.

Allele frequency differences compared to controls

An overview of all allele frequency data, odds ratios and p-values is given in supplementary Table 1. HLA phenotype frequencies(frequencies of allele carriers) including odds ratios and p-values are given in supplementary Table 2. This paper focuses on the HLA allele frequency analysis and in most cases similar results were observed for the HLA phenotype frequency analysis.

In the cHL patient group the allele frequency of the HLA-A68(28), HLA-DR11 (5) and HLA-DR15(2) was significantly increased as compared to the controls(5. 0% vs 0. 9%; $p<10^{-10}$, 11. 5% vs 0. 7%; $p<10^{-10}$, and 21% vs 14. 3%; $p=9.8\times10^{-7}$, respectively). In contrast, HLA-DR4 and HLA-DR7 allele frequencies were significantly decreased(13% vs 18%; $p=3.1\times10^{-4}$, and 5. 2% vs 11%; $p=1.4\times10^{-6}$ respectively)(Table 2; Figure 1). The associations for HLA-DR4, HLA-DR7 and HLA-DR15(2) were also observed in the EBV- cHL subgroup and not in the EBV+cHL group(see below)(Figure 1).

In EBV- cHL patients a significantly decreased allele frequency was found for HLA-DR4 and HLA-DR7(12% vs 18%; $p=6.3\times10^{-4}$, 5. 6% vs 11%; $p=1.9\times10^{-4}$) as

compared to the controls and a significantly increased frequency for HLA-A68 (28), HLA-DR11(5) and HLA-DR15(2) compared to controls(5. 6% vs 0. 9%; $p < 10^{-10}$, 13% vs 0. 7%; $p < 10^{-10}$, 23% vs 14%; $p = 8.4 \times 10^{-7}$)(Table 3, figure 1). The association for HLA-DR4, HLA-DR7 and HLA-DR15(2) are also observed in the total cHL subgroup, but not in the EBV+group, whereas the associations for HLA-A68(28) and HLA-DR11(5) are also observed in the EBV+cHL(see below).

For the EBV+cHL subgroup strong associations were observed for HLA-A1, HLA-A2, HLA-A68(28), HLA-B37, HLA-DR10 and HLA-DR11(5)(Table 3; Figure 1). The HLA-A1 frequency was increased compared to the controls(33% vs 19%; $p = 4.6 \times 10^{-6}$), whereas the HLA-A2 frequency was decreased(16% vs 34%; $p = 2.3 \times 10^{-6}$) in EBV+cHL. For HLA-A68(28)(3. 2 vs 0. 9; $p = 2.1 \times 10^{-3}$), HLA-B37(8. 4% vs 1. 9%; $p = 2.4 \times 10^{-10}$) and HLA-DR10(3. 8% vs 1. 0%; $p = 4.7 \times 10^{-4}$) an increased frequency was observed in the EBV+cHL patients as compared to the controls. For HLA-DR11(5) the allele frequency was decreased in the EBV+cHL population as compared to the controls(4. 5% vs 12%; $p = 1.3 \times 10^{-7}$). The associations for HLA-A1, HLA-A2, HLA-B37 and HLA-DR10 were observed only in the EBV+cHL subgroup and not in the EBV- cHL subgroup.

Table 1 Distribution of sex, histological subtype and age in cHL population stratified by EBV status

	All patients		EBV-Positive		EBV-Negative	
	n	%	n	%	n	%
Sex						
Male	177	52	60	77	107	46
Female	161	48	18	23	126	54
Histological subtype						
NS	256	87	46	69	192	92
MC	29	10	18	27	10	5
LR	10	3	3	4	7	3
NOS	43		11		24	
Median age (range)	35 (13-81)		37 (17-70)		32 (13-81)	

Table 2 HLA allele frequencies of HLA-A, -B, and-DR alleles with a (nearly) significant difference between controls and cHL patients

	Controls		cHL patients		Controls vs cHL
	n	%	n	%	p *
HLA-A68(28)	124	0. 9	34	5. 0	$<10^{-10}$
HLA-B51(5)	745	5. 1	52	8. 0	1.2×10^{-3}
HLA-B60(40)	1037	7. 1	26	4. 0	2.3×10^{-3}
HLA-DR4	2146	18. 1	85	12. 6	3.1×10^{-4}
HLA-DR7	1320	11. 1	35	5. 2	1.4×10^{-6}
HLA-DR11(5)	87	0. 7	77	11. 5	$<10^{-10}$
HLA-DR15(2)	1690	14. 3	142	21. 1	9.8×10^{-7}

* Significant differences ($p<0.001$) are shown in bold, suggestive ones ($p<0.003$) in italic

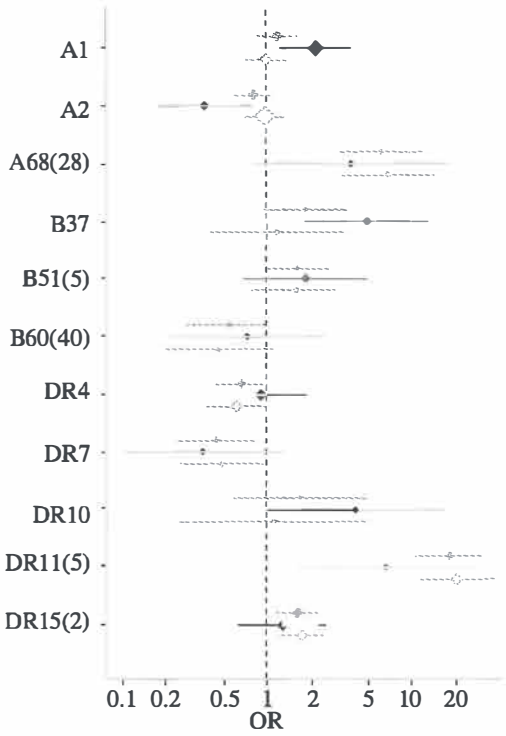

Figure 1 Odds ratios and 99. 9% confidence intervals of the phenotype allele frequencies. It shows the (nearly) significant differences between the controls and either the total cHL patient group (grey), the EBV- (white), or the EBV+(black) subgroup of patients. The size of the diamond reflects the allele frequency.

Table 3 Allele frequencies of *HLA-A*, *HLA-B*, and *HLA-DR* alleles with (nearly) significant difference between controls and EBV+or EBV- cHL subgroups

	Controls		EBV+cHL		EBV-cHL		Controls vs EBV+	Controls vs EBV-
	n	%	n	%	n	%	p*	p*
HLA-A1	2667	18. 8	52	33. 3	85	13. 0	4.0×10^{-6}	ns
HLA-A2	4828	34. 0	25	16. 0	153	37. 0	2.3×10^{-6}	ns
HLA-A68(28)	124	0. 9	5	3. 2	26	5. 6	$<10^{-10}$	$<10^{-10}$
HLA-B37	277	1. 9	13	8. 4	10	2. 2	6.3×10^{-9}	ns
HLA-B51(5)†	745	5. 1	14	7. 4	33	9. 1	ns	ns

continued

	Controls		EBV+cHL		EBV-cHL		Controls vs EBV+	Controls vs EBV-
	n	%	n	%	n	%	p*	p*
HLA-B60(40)	1037	7. 1	8	5. 2	15	3. 4	ns	2.2×10^{-3}
HLA-DR4	2146	18. 1	26	16. 7	55	11. 9	ns	6.3×10^{-4}
HLA-DR7	1320	11. 1	7	4. 5	26	5. 6	ns	1.9×10^{-4}
HLA-DR10	118	1. 0	6	3. 8	5	1. 1	4.7×10^{-4}	ns
HLA-DR11(5)	87	0. 7	7	4. 5	60	13. 0	1.3×10^{-7}	$<10^{-10}$
HLA-DR15(2)	1690	14. 3	27	17. 3	104	22. 5	ns	8.4×10^{-7}

* Significant differences (p<0. 001) are shown in bold, suggestive ones (p<0. 003) in italic.

†HLA-B51 is included because it had significantly different frequencies in the total group of cases as compared to controls (Table 2).

Differences in HLA allele frequencies between EBV+ and EBV- cHL patients

Comparison of the HLA allele frequencies revealed four significant differences between the EBV + and EBV- cHL subgroups (supplementary Table 1 and supplementary Table 2 for the phenotype frequencies). The HLA-A1 frequency was significantly increased (33% vs 13%; $p = 9.2\times10^{-5}$) and the HLA-A2 frequency was significantly decreased(16% vs 37%; $p=5.2\times10^{-5}$) consistent with previous publications. In addition, we observed a significantly increased frequency for HLA-B37(8. 4% vs 2. 2%; $p=5.5\times10^{-4}$) and HLA-Cw6 (14% vs 5. 9%; $p=2.2\times10^{-3}$) in EBV+cHL patients versus EBV- cHL patients(Table 4).

Table 4 HLA allele frequencies with a(nearly) significant difference between EBV+and EBV- cHL patients

	EBV+cHL		EBV-cHL		p-value
	n	%	n	%	
HLA-A1	52	33. 3	85	13. 0	9.2×10^{-5}
HLA-A2	25	16. 0	153	34. 0	5.2×10^{-5}
HLA-B37	13	8. 4	10	2. 2	5.5×10^{-4}
HLA-C6	21	13. 6	27	5. 9	2.2×10^{-3}

* Significant differences ($p<0.001$) are shown in bold, suggestive ones ($p<0.003$) in italic.

Association analysis of individual PCR-SSOPs in EBV+ and EBV- cHL patients

Analysis of each of the 358 PCR-SSOPs revealed a significant difference between EBV- and EBV + cHL patients for 19 HLA-A gene PCR-SSOPs (Figure 2; Supplemental Table 3). Ten of the PCR-SSOPs were specific for the HLA-A*01 allele and were more common in EBV+cHL patients, while the other nine PCR-SSOPs specific for the HLA-A*02 allele were less common in this patient group (Table 2). The 19 SSOPs with significant differences contained polymorphic residues within eight of the HLA-A*02-specific and ten of the HLA-A*01 specific SSOPs that are located at key positions in the peptide binding pockets(18)(Table 5). PCR-SSOPs that were less specific for the HLA-A*01 or HLA-A*02 alleles showed a similar trend in the odds ratio as the significant PCR-SSOPs but with lower p-values(supplementary Table 3).

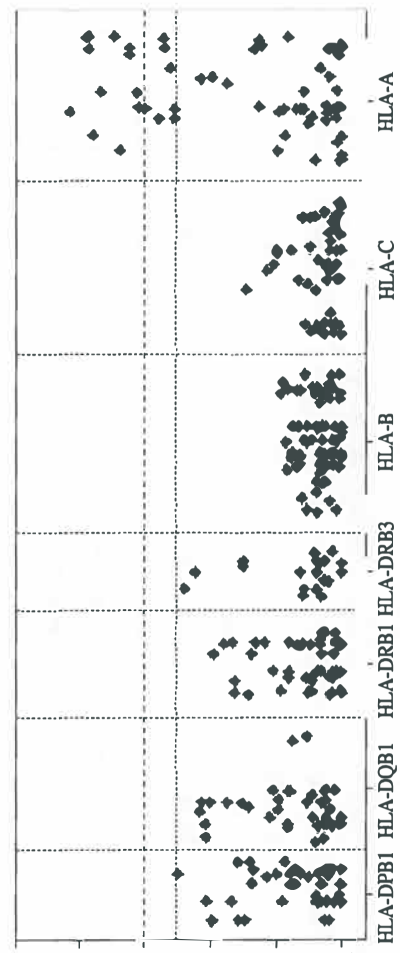

Figure 2 Genetic association of individual PCR-SSOP in EBV+ cHL The p-value of each SSOP for differences in frequencies between EBV+and EBV-cHL cases is plotted on the y-axis. Genes are ordered according to their relative positions on the short arm of chromosome 6 (6p-telomere to 6p-centromere). Strong associations with EBV status were present only for part of the PCR-SSOPs within the *HLA-A* gene.

Table 5 Overview of PCR-SSOPs with significant differences between EBV+ and EBV-cHL population

SSOP	Specificity of probe[a]	AA positions (IMGT)	Potential contact position[†]	EBV+ (%)	EBV- (%)	P	OR
c279	A*02	11-14,23-25	Peptide (24)	23.0	50.0	4.3×10^{-4}	0.32
c348	A*02,A68,A*69	141-142,144-145	TCR (145)	34.6	60.3	6.0×10^{-4}	0.37
c373	A*02,A*24,A*68,A*69	143-145,150-153	Peptide (143,152),TCR (145,150,151)	34.6	60.3	6.0×10^{-4}	0.37
c349	A*02	94-98	Peptide(95,97)	26.9	53.0	7.6×10^{-4}	0.36
c273	A*02,A*31,A*33	69-72,76-79	Peptide (70,77),TCR (69,72,76,79)	31.6	58.2	8.1×10^{-4}	0.37
c339	A*02,A*24,A*68,A*69	150-154	Peptide (152),TCR (150,151,154)	48.7	71.6	2.0×10^{-3}	0.41
C397	A02,A*23,A*24,A*68,A*69	125-128		51.3	72.8	2.5×10^{3}	0.41
c208	A*02	60,62-63,65	Peptide (62,63),TCR (62,65)	29.5	53.0	2.9×10^{-3}	0.41
c241	A*02	70,73-74,76	Peptide (70,73 74),TCR (76)	29.5	53.0	2.9×10^{-3}	0.41
c295	A*01,A*26,A*29	70-74	Peptide (70,73,74),TCR (72)	80.8	53.9	7.2×10^{-5}	3.79
c395	A*01,A*03,A*11,A*30	93-95,97-98	Peptide (95,97)	84.6	61.2	2.1×10^{-4}	3.79
c378	A*01,A*24,A*23	156,158,166-167	Peptide (156,167),TCR (158,166,167)	56.4	29.9	1.3×10^{-4}	3.05
c325	A*01	150-152	Peptide (152)TCR (150,151)	56.4	30.2	1.4×10^{-4}	3.03
c375	A*01,A*25,A*26,A66	161-163,166-168	Peptide (163,167),TCR (162,163,166,167)	56.4	30.2	1.4×10^{-4}	3.03
c281	A*01	41-44		55.8	29.3	1.7×10^{-4}	3.02
c331	A*01,A*24,A*23	164,166-168	Peptide (167),TCR (166,167)	71.8	47.0	3.4×10^{-4}	2.96
c215	A*01,A*26,A*29	74-77	Peptide (74,77),TCR (76)	59.0	35.5	1.0×10^{-3}	2.58
c211	A*01,A*03,A*11,A*30,A*31	60,62-64	Peptide (62,63),TCR (62)	87.2	68.0	1.6×10^{-3}	3.36
c332	A*01,A*11,A*24,A*26	160-163,165	Peptide (163),TCR (162,163)	66.7	45.9	2.0×10^{-3}	2.49

[a]Only CWD alleles were included;[†]Contact position according to Bjorkman 1990 (18)

Discussion

Inherited variations within the HLA genes as well as variations in host immune responses have long been recognized to be associated with susceptibility to disease, including cHL(21). A number of HLA genes, alleles and serotypes have previously been reported to be involved in the pathogenesis of cHL(5, 6). In this study we observed multiple associations that are specific for the EBV+ , the EBV- or the total cHL subpopulations. HLA-A68(28) and HLA-DR11(5) are risk alleles for cHL irrespective of the EBV status. HLA-DR4 and HLA-DR7 are associated with decreased susceptibility and HLA-DR15(2) with an increased risk in the EBV- cHL groups. In the EBV+cHL population an increased susceptibility was observed for HLA-A1, HLA-B37 and HLA-DR10, whereas resistance to disease development was observed for HLA-A2.

The previously reported association of the HLA-A gene with EBV+cHL(5, 14, 15) was confirmed both in comparison to EBV- cHL and in comparison to healthy controls with an increased risk for HLA-A*01 and a reduced risk for HLA-A*02. A tentative explanation for this association is the known presence of multiple cytotoxic T cell epitopes for LMP2 derived antigenic peptides restricted through HLA-A*02(8), whereas HLA-A*01 restricted epitopes to the latent EBV peptides have not been found (22). Analysis of the individual PCR-SSOPs revealed a significant difference for 19 out of the 358 probes in EBV+as compared to EBV- cHL. These probes discriminated between the HLA-A*01 and HLA-A*02 alleles and showed only a limited number of cross reacting other alleles. The other, less significant HLA-A*01 and HLA-A*02 identifying probes showed a similar risk pattern. The less significant level of these probes can be explained by their broader specificities including some common HLA-A alleles. For example, several probes share specificity for both HLA-A1 and HLA-A11. HLA-A*11 was previously reported to be a protective allele in EBV-associated undifferentiated

nasopharyngeal carcinoma(UNPC) in both endemic and non-endemic regions(23, 24). UNPC has the same EBV latency pattern as cHL with expression of only LMPs and EBNA1 and it was suggested that HLA-A*11 can efficiently present antigenic peptides from these proteins(24, 25). Therefore, specificity of a probe for both HLA-A*11 and HLA-A*01 might diminish the strength of its association with EBV+ cHL. Overall, our genotype analysis clearly demonstrated that not the individual probes but the complete HLA-A*01 and HLA-A*02 alleles are important for this association. The allele frequency analysis supported the importance of HLA-A1 and HLA-A2 in the association with EBV+cHL.

The strong linkage disequilibrium observed in the HLA region might affect the results of our HLA association studies. The HLA-A1 antigen is known to be in strong linkage disequilibrium with HLA-B8 and in contrast to HLA-A1, HLA-B8 is capable of presenting EBV-derived peptides(26). We observed that in the EBV+ cHL population 53% (23/43) of the HLA-A1 positive patients also possessed the HLA-B8 allele, whereas in the EBV- cHL population this percentage was 66% (46/70). In theory, presence of HLA-B8 in HLA-A1 carrying EBV + cHL, might overcome the less effective presentation of EBV derived peptides by HLA-A1. Nevertheless we saw a very significant effect for HLA-A in this study consistent with previous studies(5, 14, 15), and no significant differences for HLA-B8.

The predisposing effects of HLA-B37 and HLA-DR10 for EBV+cHL might at least be partly attributed to their strong linkage disequilibrium with HLA-A1 since HLA-A1-B37-Cw6-DR10-DQ5 is a rather common haplotype in the Caucasian population. In our study, 13 of 14 HLA-B37+ and five of six HLA-DR10+EBV+cHL patients also carried HLA-A1. Although we do not have haplotype data available, seven patients were found to potentially possess the HLA-A1-B37-DR10 haplotype, in which five were EBV+.

Two associations(HLA-A68(28) and HLA-DR11(5)) were present in both the EBV- and EBV+cHL subgroups and are thus specific for the total cHL subgroup

irrespective of the EBV status. These associations have not been reported previously. An association with HLA-DR5 has been reported previously by Robertson et al(27) in familial cHL. Significant effects of HLA-A1, HLA-B8 and HLA-B18 for cHL that have been reported in previous publications(11-13) could not be confirmed in the total patient group in our study, whereas the previously reported association with HLA-B5(28) was borderline significant in our study. These differences might be explained by differences in sample size, patient selection, and proportion of EBV+cases. The previous studies were carried out in relatively small numbers of patients ranging from 11 to 137 within different countries such as Egypt, Czech Republic, Sweden, Denmark, and United States. Distribution of HLA alleles varies widely by ethnicity and geography, which might lead to differences in disease-associated HLA alleles among different ethnic groups and geographic locations. In addition most case-control studies used low-resolution serological HLA typing. A limitation of this approach is the inability to discriminate between specific allele variants, which might be more or less common in different populations.

The HLA-DR4, HLA-DR7 and HLA-DR15(2) class II associations were not observed in the EBV+subgroup and represent associations specific for the EBV- cHL subgroup. The HLA-A1 and HLA-A2 class I associations were specific for the EBV+ cHL subgroup. HLA class II molecules mainly function as binding and presenting exogenous antigenic peptides to CD4+ helper T cells, whereas HLA class I is known to bind and present peptides derived from endogenous proteins to CD8+ cytotoxic T lymphocytes (29). Our findings thus support a differential immunogenic basis with a more prominent role in the effector phase of the immune response for HLA class I in EBV+cHL and an immunoregulatory role for HLA class II in the pathogenesis of EBV- cHL. This suggests presence of a specific HLA class II restricted antigen involved in the pathogenesis of EBV- cHL. Whether this is a pathogen-derived antigen or an antigen derived from a mutated

protein remains unknown.

In conclusion, the current study confirms the previously reported genetic association of the HLA-A1 and HLA-A2 in EBV+cHL. The genetic influence of these two HLA antigens is more important than individual SNPs that might be shared by multiple HLA-A alleles. In addition, we demonstrated two significant associations for the cHL population, three significant HLA class II associations specific for the EBV- cHL population and one additional association for the EBV + cHL subgroup. This implies an influence of the interaction between environmental and genetic risk factors in the development of cHL and supports heterogeneity in the genetic predisposition to EBV+and EBV- cHL.

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Table S1 HLA allele frequency analysis including OR and p-values

	Allele frequency										EBV+ versus EBV-				EBV+ versus controls				EBV- versus controls				all cases versus controls			
	EBV+		EBV-		EBV?		All cases		Controls		OR	99.9% CI		p-value	OR	99.9% CI		p-value	OR	99.9% CI		p-value	OR	99.9% CI		p-value
HLA-A	156	%	464	%	54	%	674	%	14198	%																
A1	52	33.3	85	18.3	7	13.0	144	21.4	2667	18.8	2.23	1.12	4.42	9.2E-05	2.16	1.23	3.80	4.0E-06	0.97	0.65	1.45	8.0E-01	1.17	0.86	1.61	9.5E-02
A2	25	16.0	153	33.0	20	37.0	198	29.4	4828	34.0	0.39	0.18	0.85	5.2E-05	0.37	0.18	0.76	2.3E-06	0.95	0.69	1.33	6.4E-01	0.81	0.61	1.07	1.3E-02
A3	29	18.6	64	13.8	11	20.4	104	15.4	2524	17.8	1.43	0.64	3.21	1.5E-01	1.06	0.53	2.09	7.9E-01	0.74	0.47	1.16	2.7E-02	0.84	0.59	1.21	1.2E-01
A11	8	5.1	31	6.7	0	0.0	39	5.8	737	5.2	0.76	0.20	2.89	4.9E-01	0.99	0.30	3.28	9.7E-01	1.31	0.70	2.44	1.6E-01	1.12	0.64	1.96	5.0E-01
A23	3	1.9	4	0.9	0	0.0	7	1.0	151	1.1	2.25	0.18	28.36	2.8E-01	1.82	0.26	12.66	3.0E-01	0.81	0.15	4.32	6.8E-01	0.98	0.27	3.51	9.5E-01
A24	14	9.0	45	9.7	6	11.1	65	9.6	1259	8.9	0.92	0.32	2.64	7.9E-01	1.01	0.40	2.56	9.6E-01	1.10	0.65	1.87	5.4E-01	1.10	0.71	1.70	4.9E-01
A25	2	1.3	6	1.3	1	1.9	9	1.3	83	0.6	0.99	0.07	14.81	9.9E-01	2.21	0.21	23.62	2.6E-01	2.23	0.55	9.03	5.3E-02	2.30	0.72	7.36	1.5E-02
A26	4	2.6	9	1.9	0	0.0	13	1.9	259	1.8	1.33	0.18	9.84	6.4E-01	1.42	0.26	7.60	4.9E-01	1.06	0.35	3.28	8.6E-01	1.06	0.41	2.72	8.4E-01
A29	3	1.9	9	1.9	1	1.9	13	1.9	358	2.5	0.99	0.11	9.08	9.9E-01	0.76	0.11	5.20	6.3E-01	0.76	0.25	2.35	4.3E-01	0.76	0.30	1.94	3.4E-01
A30	1	0.6	0	0.0	0	0.0	1	0.1	185	1.3	nd	nd	nd	nd	0.49	0.02	13.38	4.7E-01	nd	nd	nd	nd	0.11	0.00	3.06	8.4E-03
A31	1	0.6	17	3.7	1	1.9	19	2.8	405	2.9	0.17	0.01	5.08	5.2E-02	0.22	0.01	5.99	9.8E-02	1.30	0.56	2.97	3.0E-01	0.99	0.45	2.16	9.6E-01
A32	6	3.8	13	2.8	4	7.4	23	3.4	491	3.5	1.39	0.27	7.25	5.1E-01	1.12	0.28	4.43	7.9E-01	0.80	0.31	2.06	4.4E-01	0.99	0.48	2.01	9.5E-01
A68	5	3.2	26	5.6	3	5.6	34	5.0	124	0.9	0.56	0.11	2.87	2.3E-01	3.76	0.82	17.27	2.1E-03	6.74	3.25	13.95	<10E-10	6.03	3.15	11.56	<10E-10
Others	3	1.9	2	0.4	0	0.0	5	0.7	127	0.9	4.53	0.22	92.76	7.1E-02	2.17	0.31	15.13	1.8E-01	0.48	0.05	5.03	2.9E-01	0.83	0.18	3.73	6.8E-01
HLA-B	154	%	446	%	50	%	650	%	14566	%																
B7	28	18.2	92	20.6	11	22.0	131	20.2	2449	16.8	0.86	0.39	1.88	5.1E-01	1.10	0.55	2.20	6.5E-01	1.29	0.87	1.90	3.4E-02	1.25	0.90	1.74	2.6E-02
B8	27	17.5	65	14.6	5	10.0	97	14.9	2073	14.2	1.25	0.55	2.84	3.8E-01	1.28	0.64	2.58	2.4E-01	1.03	0.66	1.61	8.4E-01	1.06	0.73	1.53	6.2E-01

continued

	Allele frequency										EBV+ versus EBV-				EBV+ versus controls				EBV- versus controls				all cases versus controls			
	EBV+		EBV-		EBV?		All cases		Controls		OR	99.9%CI		p-value	OR	99.9% CI		p-value	OR	99.9% CI		p-value	OR	99.9%CI		p-value
B13	0	0.0	5	1.1	0	0.0	5	0.8	315	2.2	nd	nd	nd	nd	nd	nd	nd	nd	0.51	0.12	2.28	1.3E-01	0.35	0.08	1.55	1.5E-02
B14	1	0.6	2	0.4	1	2.0	4	0.6	225	1.5	1.45	0.03	82.61	7.6E-01	0.42	0.02	11.39	3.7E-01	0.29	0.03	2.99	6.2E-02	0.39	0.07	2.09	5.7E-02
B18	4	2.6	17	3.8	6	12.0	27	4.2	472	3.2	0.67	0.11	4.30	4.8E-01	0.80	0.15	4.25	6.5E-01	1.18	0.52	2.71	5.0E-01	1.29	0.67	2.52	2.0E-01
B27	1	0.6	15	3.4	1	2.0	17	2.6	620	4.3	0.19	0.01	5.70	7.1E-02	0.15	0.01	4.00	2.7E-02	0.78	0.33	1.88	3.6E-01	0.60	0.27	1.37	4.1E-02
B35	12	7.8	49	11.0	4	8.0	65	10.0	1276	8.8	0.68	0.23	2.07	2.6E-01	0.88	0.33	2.38	6.7E-01	1.29	0.77	2.14	1.0E-01	1.16	0.74	1.80	2.8E-01
B37	13	8.4	10	2.2	0	0.0	23	3.5	277	1.9	4.02	0.97	16.64	5.5E-04	4.76	1.79	12.60	6.3E-09	1.18	0.41	3.45	6.1E-01	1.89	0.92	3.91	3.3E-03
B39	3	1.9	7	1.6	0	0.0	10	1.5	306	2.1	1.25	0.13	12.33	7.5E-01	0.93	0.13	6.37	9.0E-01	0.74	0.21	2.64	4.4E-01	0.73	0.25	2.11	3.3E-01
B41	1	0.6	5	1.1	1	2.0	7	1.1	104	0.7	0.58	0.02	21.48	6.1E-01	0.91	0.03	25.07	9.2E-01	1.58	0.35	7.17	3.2E-01	1.51	0.42	5.51	2.9E-01
B44	12	7.8	39	8.7	4	8.0	55	8.5	1697	11.7	0.88	0.28	2.74	7.1E-01	0.64	0.24	1.73	1.4E-01	0.73	0.42	1.27	5.9E-02	0.70	0.44	1.12	1.3E-02
B49	2	1.3	5	1.1	1	2.0	8	1.2	85	0.6	1.16	0.07	18.53	8.6E-01	2.24	0.21	23.96	2.5E-01	1.93	0.42	8.85	1.5E-01	2.12	0.62	7.22	3.8E-02
B51	14	9.1	33	7.4	5	10.0	52	8.0	745	5.1	1.25	0.42	3.75	5.0E-01	1.86	0.73	4.70	2.6E-02	1.48	0.81	2.72	3.2E-02	1.61	0.99	2.64	1.2E-03
B55	0	0.0	6	1.3	0	0.0	6	0.9	252	1.7	nd	nd	nd	nd	nd	nd	nd	nd	0.77	0.20	3.04	5.4E-01	0.53	0.14	2.07	1.2E-01
B57	7	4.5	7	1.6	0	0.0	14	2.2	423	2.9	2.99	0.50	17.83	3.5E-02	1.59	0.44	5.75	2.3E-01	0.53	0.15	1.89	9.6E-02	0.74	0.30	1.82	2.6E-01
B60	8	5.2	15	3.4	3	6.0	26	4.0	1037	7.1	1.57	0.36	6.88	3.1E-01	0.71	0.22	2.37	3.5E-01	0.45	0.19	1.08	2.2E-03	0.54	0.28	1.06	2.3E-03
B61	4	2.6	11	2.5	1	2.0	16	2.5	197	1.4	1.05	0.15	7.39	9.3E-01	1.95	0.36	10.47	1.9E-01	1.84	0.66	5.18	4.7E-02	1.84	0.77	4.38	1.9E-02
B62	10	6.5	40	9.0	4	8.0	54	8.3	1273	8.7	0.70	0.21	2.36	3.4E-01	0.73	0.25	2.14	3.3E-01	1.03	0.59	1.79	8.7E-01	0.95	0.59	1.53	7.0E-01
Others	7	4.5	23	5.2	3	6.0	33	5.1	740	5.1	0.88	0.20	3.75	7.6E-01	0.89	0.25	3.20	7.6E-01	1.02	0.50	2.08	9.4E-01	1.00	0.55	1.82	1.0E+00
HLA-C	154	%	454	%	46	%	654	%																		
C1	0	0.0	15	3.3	0	0.0	15	2.3			nd	nd	nd	nd												

continued

	Allele frequency									EBV+ versus EBV-				EBV+ versus controls			EBV- versus controls			all cases versus controls		
	EBV+		EBV-		EBV?		All cases		Controls	OR	99.9% CI		p-value	OR	99.9% CI	p-value	OR	99.9% CI	p-value	OR	99.9% CI	p-value
C2	7	4.5	25	5.5	1	2.2	33	5.0		0.82	0.19	3.46	6.4E-01									
C3	20	13.0	69	15.2	6	13.0	95	14.5		0.83	0.34	2.05	5.0E-01									
C4	16	10.4	54	11.9	6	13.0	76	11.6		0.86	0.32	2.31	6.1E-01									
C5	8	5.2	30	6.6	2	4.3	40	6.1		0.77	0.20	2.98	5.3E-01									
C6	21	13.6	27	5.9	1	2.2	49	7.5		2.50	0.91	6.87	2.2E-03									
C7	58	37.7	184	40.5	23	50.0	265	40.5		0.89	0.47	1.67	5.3E-01									
C8	5	3.2	11	2.4	1	2.2	17	2.6		1.35	0.22	8.19	5.8E-01									
C12	4	2.6	11	2.4	2	4.3	17	2.6		1.07	0.15	7.52	9.0E-01									
C14	3	1.9	8	1.8	2	4.3	13	2.0		1.11	0.12	10.50	8.8E-01									
C15	8	5.2	13	2.9	1	2.2	22	3.4		1.86	0.41	8.43	1.7E-01									
C16	3	1.9	3	0.7	1	2.2	7	1.1		2.99	0.20	44.64	1.6E-01									
C17	1	0.6	4	0.9	0	0.0	5	0.8		0.74	0.02	29.50	7.8E-01									
HLA-DQ	150	%	454	%	52	%	656	%														
DQB2	25	16.7	71	15.6	9	17.3	105	16.0		1.08	0.47	2.49	7.7E-01									
DQB4	8	5.3	7	1.5	1	1.9	16	2.4		3.60	0.64	20.34	9.7E-03									
DQB5	30	20.0	69	15.2	10	19.2	109	16.6		1.39	0.63	3.10	1.7E-01									
DQB6	49	32.7	169	37.2	17	32.7	235	35.8		0.82	0.42	1.58	3.1E-01									
DQB7	15	10.0	90	19.8	13	25.0	118	18.0		0.45	0.17	1.19	5.9E-03									
DQB8	21	14.0	32	7.0	2	3.8	55	8.4		2.15	0.80	5.73	9.1E-03									

continued

	Allele frequency										EBV+ versus EBV-				EBV+ versus controls				EBV- versus controls				all cases versus controls			
	EBV+		EBV-		EBV?		All cases		Controls		OR	99.9% CI		p-value	OR	99.9% CI		p-value	OR	99.9% CI		p-value	OR	99.9% CI		p-value
DQB9	2	1.3	16	3.5	0	0.0	18	2.7			0.37	0.03	4.45	1.7E-01												
HLA-DR	156	%	462	%	54	%	672	%	11844	%																
DR1	17	10.9	46	10.0	8	14.8	71	10.6	1501	12.7	1.11	0.41	2.97	7.4E-01	0.84	0.36	1.97	5.1E-01	0.76	0.45	1.28	8.4E-02	0.81	0.53	1.24	1.1E-01
DR3	24	15.4	60	13.0	7	13.0	91	13.5	1871	15.8	1.22	0.52	2.88	4.5E-01	0.97	0.46	2.02	8.9E-01	0.80	0.50	1.26	1.0E-01	0.83	0.57	1.22	1.2E-01
DR4	26	16.7	55	11.9	4	7.4	85	12.6	2146	18.1	1.48	0.63	3.46	1.3E-01	0.90	0.44	1.84	6.4E-01	0.61	0.38	0.99	6.3E-04	0.65	0.44	0.97	3.1E-04
DR7	7	4.5	26	5.6	2	3.7	35	5.2	1320	11.1	0.79	0.19	3.31	5.8E-01	0.37	0.10	1.34	8.4E-03	0.48	0.24	0.93	1.9E-04	0.44	0.25	0.78	1.4E-06
DR8	8	5.1	9	1.9	1	1.9	18	2.7	429	3.6	2.72	0.53	13.87	3.6E-02	1.44	0.43	4.80	3.2E-01	0.53	0.17	1.62	5.7E-02	0.73	0.33	1.63	2.0E-01
DR9	0	0.0	5	1.1	0	0.0	5	0.7	187	1.6	nd	nd	nd	nd	nd	nd	nd	nd	0.68	0.15	3.05	4.0E-01	0.47	0.10	2.09	8.7E-02
DR10	6	3.8	5	1.1	0	0.0	11	1.6	118	1.0	3.66	0.49	27.46	2.4E-02	3.97	0.98	16.17	4.7E-04	1.09	0.24	4.92	8.6E-01	1.65	0.58	4.71	1.1E-01
DR11	7	4.5	60	13.0	10	18.5	77	11.5	87	0.7	0.31	0.08	1.22	3.2E-03	6.35	1.69	23.79	1.3E-07	20.17	11.33	35.91	<10E-10	17.49	10.26	29.80	<10E-10
DR12	2	1.3	6	1.3	1	1.9	9	1.3	283	2.4	0.99	0.07	14.75	9.9E-01	0.53	0.05	5.56	3.7E-01	0.54	0.14	2.11	1.3E-01	0.55	0.18	1.70	7.9E-02
DR13	24	15.4	68	14.7	8	14.8	100	14.9	1613	13.6	1.05	0.45	2.46	8.4E-01	1.15	0.55	2.41	5.2E-01	1.09	0.70	1.70	5.0E-01	1.11	0.77	1.60	3.5E-01
DR14	6	3.8	16	3.5	2	3.7	24	3.6	384	3.2	1.12	0.22	5.55	8.2E-01	1.19	0.30	4.75	6.7E-01	1.07	0.46	2.52	7.9E-01	1.11	0.55	2.24	6.4E-01
DR15	27	17.3	104	22.5	11	20.4	142	21.1	1690	14.3	0.72	0.33	1.58	1.7E-01	1.26	0.62	2.54	2.8E-01	1.75	1.20	2.54	8.4E-07	1.61	1.17	2.22	9.8E-07
Others	2	1.3	2	0.4	0	0.0	4	0.6	215	1.8	2.99	0.11	81.37	2.5E-01	0.70	0.07	7.39	6.2E-01	0.24	0.02	2.45	2.7E-02	0.32	0.06	1.71	1.9E-02
HLA-DP	156	%	462	%	54	%	672	%																		
DP01:01	4	2.6	20	4.3	4	7.4	28	4.2			0.58	0.09	3.62	3.2E-01												
DP02:01	26	16.7	48	10.4	3	5.6	77	11.5			1.73	0.72	4.10	3.7E-02												

continued

	Allele frequency								EBV+ versus EBV-				EBV+ versus controls			EBV- versus controls			all cases versus controls		
	EBV+		EBV-		EBV?		All cases	Controls	OR	99.9% CI		p-value	OR	99.9% CI	p-value	OR	99.9% CI	p-value	OR	99.9% CI	p-value
DP04:02 /105:01	5	3.2	41	8.9	7	13.0	53	7.9	0.34	0.07	1.67	2.0E-02									
DP03:01	22	14.1	85	18.4	12	22.2	119	17.7	0.73	0.31	1.71	2.2E-01									
DP04:01	79	50.6	194	42.0	21	38.9	294	43.8	1.42	0.77	2.61	6.0E-02									
DP05:01	3	1.9	8	1.7	0	0.0	11	1.6	1.11	0.12	10.55	8.8E-01									
DP06:01	3	1.9	5	1.1	3	5.6	11	1.6	1.79	0.16	20.21	4.2E-01									
DP10:01	1	0.6	5	1.1	1	1.9	7	1.0	0.59	0.02	21.96	6.3E-01									
DP13:01	4	2.6	9	1.9	0	0.0	13	1.9	1.32	0.18	9.80	6.4E-01									
DP14:01	1	0.6	14	3.0	0	0.0	15	2.2	0.21	0.01	6.31	9.4E-02									
DP19:01	5	3.2	8	1.7	0	0.0	13	1.9	1.88	0.28	12.58	2.7E-01									
Others	3	1.9	25	5.4	3	5.6	31	4.6	0.34	0.04	2.62	7.0E-02									

* Allelic frequency < 1% was excluded

Table S2 HLA phenotype frequencies including OR and p-value

	HLA Phenotype										EBV+ versus EBV-				EBV+ versus controls				EBV- versus controls				all cases versus controls			
	EBV+		EBV-		EBV?		All cases		Controls		OR	99.9%CI		p-value	OR	99.9%CI		p-value	OR	99.9%CI		p-value	OR	99.9%CI		p-value
HLA-A	**78**	**%**	**232**	**%**	**27**	**%**	**337**	**%**	**7099**	**%**																
A1	43	55.1	70	30.2	7	25.9	120	35.6	2426	34.17	2.84	1.17	6.89	7.4E-05	2.37	1.11	5.03	1.1E-04	0.83	0.52	1.34	2.1E-01	1.07	0.73	1.56	5.9E-01
A2	23	29.5	123	53.0	16	59.3	162	48.1	4052	57.08	0.37	0.15	0.93	3.2E-04	0.31	0.14	0.71	1.0E-06	0.85	0.55	1.32	2.2E-01	0.70	0.48	1.01	1.1E-03
A3	27	34.6	56	24.1	11	40.7	94	27.9	2296	32.34	1.66	0.66	4.22	7.1E-02	1.11	0.50	2.43	6.7E-01	0.67	0.40	1.11	8.4E-03	0.81	0.54	1.22	8.7E-02
A11	8	10.3	30	12.9	0	0.0	38	11.3	713	10.04	0.77	0.19	3.08	5.3E-01	1.02	0.30	3.52	9.5E-01	1.33	0.69	2.57	1.5E-01	1.14	0.64	2.04	4.6E-01
A23	3	3.8	4	1.7	0	0.0	7	2.1	151	2.13	2.28	0.18	29.23	2.8E-01	1.84	0.26	13.02	3.0E-01	0.81	0.15	4.34	6.7E-01	0.98	0.27	3.53	9.5E-01
A24	14	17.9	42	18.1	5	18.5	61	18.1	1258	17.72	0.99	0.32	3.04	9.8E-01	1.02	0.38	2.70	9.6E-01	1.03	0.58	1.82	8.8E-01	1.03	0.64	1.65	8.6E-01
A25	2	2.6	6	2.6	1	3.7	9	2.7	83	1.17	0.99	0.07	15.08	9.9E-01	2.22	0.20	24.16	2.6E-01	2.24	0.55	9.18	5.2E-02	2.32	0.72	7.47	1.5E-02
A26	4	5.1	8	3.4	0	0.0	12	3.6	259	3.65	1.51	0.19	11.91	5.1E-01	1.43	0.26	7.83	4.9E-01	0.94	0.28	3.14	8.7E-01	0.98	0.36	2.62	9.3E-01
A29	3	3.8	8	3.4	1	3.7	12	3.6	358	5.04	1.12	0.12	10.85	8.7E-01	0.75	0.11	5.27	6.3E-01	0.67	0.20	2.23	2.7E-01	0.70	0.26	1.86	2.2E-01
A30	1	1.3	0	0.0	0	0.0	1	0.3	185	2.61	nd	nd	nd	8.4E-02	0.49	0.02	13.44	4.6E-01	nd	nd	nd	1.3E-02	0.11	0.00	3.03	8.0E-03
A31	1	1.3	16	6.9	1	3.7	18	5.3	405	5.71	0.18	0.01	5.36	6.0E-02	0.21	0.01	5.91	9.3E-02	1.22	0.51	2.92	4.4E-01	0.93	0.41	2.11	7.8E-01
A32	6	7.7	13	5.6	4	14.8	23	6.8	491	6.92	1.40	0.26	7.57	5.1E-01	1.12	0.27	4.58	7.9E-01	0.80	0.31	2.07	4.4E-01	0.99	0.48	2.04	9.5E-01
A68	4	5.1	26	11.2	3	11.1	33	9.8	124	1.75	0.43	0.07	2.65	1.2E-01	3.04	0.55	16.90	2.5E-02	7.10	3.36	14.98	<10E-10	6.11	3.12	11.97	<10E-10
Others	2	2.6	2	0.9	0	0.0	4	1.2	127	1.79	3.03	0.11	83.85	2.5E-01	1.44	0.13	15.54	6.1E-01	0.48	0.05	5.02	2.9E-01	0.66	0.12	3.54	4.1E-01
HLA-B	**77**	**%**	**222**	**%**	**25**	**%**	**324**	**%**	**7283**	**%**																
B7	25	32.5	79	35.6	10	40.0	114	35.2	2233	30.7	0.87	0.35	2.19	6.2E-01	1.09	0.49	2.43	7.3E-01	1.25	0.78	2.00	1.2E-01	1.23	0.83	1.82	8.4E-02
B8	24	31.2	58	26.1	5	20.0	87	26.9	1932	26.5	1.28	0.49	3.32	3.9E-01	1.25	0.56	2.83	3.6E-01	0.98	0.59	1.63	8.9E-01	1.02	0.67	1.55	9.0E-01

continued

	HLA Phenotype										EBV+ versus EBV-				EBV+ versus controls				EBV- versus controls				all cases versus controls			
	EBV+		EBV-		EBV?		All cases		Controls		OR	99.9% CI		p-value	OR	99.9% CI		p-value	OR	99.9% CI		p-value	OR	99.9% CI		p-value
B13	0	0.0	5	2.3	0	0.0	5	1.5	311	4.3	nd	nd	nd	1.8E-01	nd	nd	nd	6.4E-02	0.52	0.12	2.32	1.4E-01	0.35	0.08	1.57	1.6E-02
B14	3	3.9	9	4.1	1	4.0	13	4.0	224	3.1	0.96	0.10	9.00	9.5E-01	1.28	0.18	8.99	6.8E-01	1.33	0.43	4.17	4.1E-01	1.32	0.51	3.43	3.4E-01
B18	4	5.2	17	7.7	6	24.0	27	8.3	465	6.4	0.66	0.10	4.34	4.7E-01	0.80	0.15	4.39	6.7E-01	1.22	0.52	2.83	4.5E-01	1.33	0.68	2.63	1.6E-01
B27	1	1.3	14	6.3	1	4.0	16	4.9	603	8.3	0.20	0.01	6.06	8.3E-02	0.15	0.01	4.01	2.6E-02	0.75	0.30	1.87	2.9E-01	0.58	0.24	1.35	3.1E-02
B35	12	15.6	43	19.4	4	16.0	59	18.2	1214	16.7	0.77	0.24	2.49	4.6E-01	0.92	0.33	2.61	8.0E-01	1.20	0.68	2.12	2.9E-01	1.11	0.69	1.81	4.7E-01
B37	13	16.9	10	4.5	0	0.0	23	7.1	270	3.7	4.31	1.00	18.57	4.4E-04	5.28	1.90	14.66	2.2E-09	1.23	0.41	3.62	5.4E-01	1.98	0.95	4.16	1.9E-03
B39	3	3.9	7	3.2	0	0.0	10	3.1	306	4.2	1.25	0.12	12.59	7.5E-01	0.92	0.13	6.48	8.9E-01	0.74	0.21	2.67	4.4E-01	0.73	0.25	2.13	3.2E-01
B41	1	1.3	4	1.8	1	4.0	6	1.9	104	1.4	0.72	0.02	29.15	7.7E-01	0.91	0.03	25.33	9.2E-01	1.27	0.23	6.88	6.5E-01	1.30	0.32	5.25	5.3E-01
B44	12	15.6	37	16.7	3	12.0	52	16.0	1693	23.2	0.92	0.28	3.04	8.3E-01	0.61	0.22	1.72	1.1E-01	0.66	0.36	1.20	2.2E-02	0.63	0.38	1.05	2.6E-03
B49	2	2.6	5	2.3	1	4.0	8	2.5	85	1.2	1.16	0.07	18.81	8.6E-01	2.26	0.21	24.52	2.5E-01	1.95	0.42	9.02	1.4E-01	2.14	0.63	7.35	3.7E-02
B51	13	16.9	31	14.0	5	20.0	49	15.1	744	10.2	1.25	0.38	4.10	5.3E-01	1.79	0.65	4.90	5.5E-02	1.43	0.74	2.73	7.1E-02	1.57	0.93	2.65	4.7E-03
B55	0	0.0	6	2.7	0	0.0	6	1.9	252	3.5	nd	nd	nd	1.5E-01	nd	nd	nd	9.7E-02	0.78	0.20	3.07	5.4E-01	0.53	0.13	2.08	1.2E-01
B57	7	9.1	7	3.2	0	0.0	14	4.3	421	5.8	3.07	0.50	18.88	3.4E-02	1.63	0.44	6.07	2.2E-01	0.53	0.15	1.90	9.6E-02	0.74	0.30	1.84	2.7E-01
B60	8	10.4	14	6.3	3	12.0	25	7.7	1037	14.2	1.72	0.37	7.94	2.4E-01	0.70	0.20	2.40	3.4E-01	0.41	0.16	1.01	7.9E-04	0.50	0.25	1.01	9.2E-04
B61	4	5.2	11	5.0	1	4.0	16	4.9	197	2.7	1.05	0.15	7.56	9.3E-01	1.97	0.36	10.86	1.8E-01	1.88	0.66	5.33	4.4E-02	1.87	0.78	4.49	1.7E-02
B62	9	11.7	38	17.1	4	16.0	51	15.7	1272	17.5	0.64	0.17	2.37	2.6E-01	0.63	0.19	2.02	1.8E-01	0.98	0.54	1.77	8.9E-01	0.88	0.53	1.47	4.2E-01
Others	7	9.1	23	10.4	3	12.0	33	10.2	739	10.1	0.87	0.19	3.85	7.5E-01	0.89	0.24	3.28	7.6E-01	1.02	0.49	2.14	9.2E-01	1.00	0.54	1.86	9.8E-01
HLA-C	**77**	**%**	**227**	**%**	**23**	**%**	**327**	**%**																		
C1	0	0.0	15	6.6	0	0.0	15	4.6			nd	nd	nd	2.1E-02												

continued

	HLA Phenotype									EBV+ versus EBV-				EBV+ versus controls			EBV- versus controls			all cases versus controls		
	EBV+		EBV-		EBV?		All cases		Controls	OR	99.9% CI		p-value	OR	99.9% CI	p-value	OR	99.9% CI	p-value	OR	99.9% CI	p-value
C2	6	7.8	24	10.6	1	4.3	31	9.5		0.71	0.15	3.43	4.8E-01									
C3	19	24.7	62	27.3	6	26.1	87	26.6		0.87	0.32	2.37	6.5E-01									
C4	15	19.5	49	21.6	6	26.1	70	21.4		0.88	0.30	2.60	7.0E-01									
C5	8	10.4	29	12.8	2	8.7	39	11.9		0.79	0.20	3.19	5.8E-01									
C6	20	26.0	26	11.5	1	4.3	47	14.4		2.71	0.91	8.12	2.1E-03									
C7	46	59.7	138	60.8	16	69.6	200	61.2		0.96	0.39	2.32	8.7E-01									
C8	5	6.5	11	4.8	1	4.3	17	5.2		1.36	0.22	8.50	5.8E-01									
C12	4	5.2	11	4.8	2	8.7	17	5.2		1.08	0.15	7.73	9.0E-01									
C14	3	3.9	8	3.5	2	8.7	13	4.0		1.11	0.11	10.76	8.8E-01									
C15	8	10.4	12	5.3	1	4.3	21	6.4		2.08	0.43	9.98	1.2E-01									
C16	3	3.9	3	1.3	1	4.3	7	2.1		3.03	0.20	46.07	1.6E-01									
C17	1	1.3	4	1.8	0	0.0	5	1.5		0.73	0.02	29.81	7.8E-01									
HLA-DQ	**75**	**%**	**227**	**%**	**26**	**%**	**328**	**%**														
DQB2	23	30.7	64	28.2	8	30.8	95	29.0		1.13	0.43	2.93	6.8E-01									
DQB4	8	10.7	7	3.1	1	3.8	16	4.9		3.75	0.64	21.90	8.8E-03									
DQB5	26	34.7	59	26.0	9	34.6	94	28.7		1.51	0.59	3.87	1.5E-01									
DQB6	38	50.7	133	58.6	16	61.5	187	57.0		0.73	0.30	1.75	2.3E-01									
DQB7	13	17.3	78	34.4	12	46.2	103	31.4		0.40	0.13	1.21	5.3E-03									
DQB8	20	26.7	28	12.3	1	3.8	49	14.9		2.58	0.87	7.66	3.2E-03									

continued

	HLA Phenotype										EBV+ versus EBV-				EBV+ versus controls				EBV- versus controls				all cases versus controls			
	EBV+		EBV-		EBV?		All cases		Controls		OR	99.9% CI		p-value	OR	99.9% CI		p-value	OR	99.9% CI		p-value	OR	99.9% CI		p-value
DQB9	2	2.7	15	6.6	0	0.0	17	5.2			0.39	0.03	4.80	2.0E-01												
HLA-DR	**78**	**%**	**231**	**%**	**27**	**%**	**336**	**%**	**5922**	**%**																
DR1	16	20.5	41	17.7	7	25.9	64	19.0	1416	23.9	1.20	0.40	3.53	5.9E-01	0.82	0.32	2.08	4.8E-01	0.69	0.39	1.22	3.1E-02	0.75	0.47	1.20	4.1E-02
DR3	22	28.2	55	23.8	7	25.9	84	25.0	1734	29.3	1.26	0.48	3.32	4.4E-01	0.95	0.41	2.18	8.4E-01	0.75	0.45	1.27	7.2E-02	0.81	0.53	1.23	9.3E-02
DR4	23	29.5	43	18.6	3	11.1	69	20.5	1969	33.2	1.83	0.68	4.91	4.3E-02	0.84	0.37	1.91	4.8E-01	0.46	0.26	0.81	3.3E-06	0.52	0.33	0.82	1.3E-06
DR7	7	9.0	25	10.8	2	7.4	34	10.1	1246	21.0	0.81	0.19	3.56	6.4E-01	0.37	0.10	1.37	9.2E-03	0.46	0.23	0.92	1.7E-04	0.42	0.23	0.77	1.4E-06
DR8	8	10.3	9	3.9	1	3.7	18	5.4	421	7.1	2.82	0.54	14.85	3.3E-02	1.49	0.43	5.16	2.8E-01	0.53	0.17	1.64	6.0E-02	0.74	0.33	1.67	2.2E-01
DR9	0	0.0	5	2.2	0	0.0	5	1.5	185	3.1	nd	nd	nd	1.9E-01	nd	nd	nd	1.1E-01	0.69	0.15	3.10	4.1E-01	0.47	0.10	2.11	8.9E-02
DR10	6	7.7	5	2.2	0	0.0	11	3.3	118	2.0	3.77	0.49	29.02	2.3E-02	4.10	0.98	17.15	4.4E-04	1.09	0.24	4.97	8.5E-01	1.66	0.58	4.78	1.1E-01
DR11	7	9.0	57	24.7	10	37.0	74	22.0	87	1.5	0.30	0.07	1.22	3.1E-03	6.61	1.71	25.54	1.1E-07	21.97	11.88	40.65	<10E-10	18.94	10.82	33.17	<10E-10
DR12	2	2.6	5	2.2	1	3.7	8	2.4	283	4.8	1.19	0.07	19.32	8.4E-01	0.52	0.05	5.59	3.6E-01	0.44	0.10	1.98	6.5E-02	0.49	0.15	1.60	4.2E-02
DR13	23	29.5	61	26.4	8	29.6	92	27.4	1612	27.2	1.17	0.45	3.02	6.0E-01	1.12	0.49	2.55	6.6E-01	0.96	0.58	1.58	7.9E-01	1.01	0.67	1.52	9.5E-01
DR14	6	7.7	16	6.9	2	7.4	24	7.1	384	6.5	1.12	0.22	5.76	8.2E-01	1.20	0.29	4.92	6.7E-01	1.07	0.45	2.56	7.9E-01	1.11	0.54	2.28	6.3E-01
DR15	23	29.5	91	39.4	10	37.0	124	36.9	1690	28.5	0.64	0.25	1.63	1.2E-01	1.05	0.46	2.38	8.5E-01	1.63	1.03	2.56	3.6E-04	1.46	1.00	2.15	1.0E-03
Others	2	2.6	2	0.9	0	0.0	4	1.2	215	3.6	3.01	0.11	83.28	2.5E-01	0.70	0.07	7.46	6.2E-01	0.23	0.02	2.43	2.5E-02	0.32	0.06	1.70	1.8E-02
HLA-DP	**78**	**%**	**231**	**%**	**27**	**%**	**336**	**%**																		
DP01:01	4	5.1	19	8.2	4	14.8	27	8.0			0.60	0.09	3.89	3.7E-01												
DP02:01	25	32.1	45	19.5	3	11.1	73	21.7			1.95	0.74	5.13	2.2E-02												

continued

	HLA Phenotype																							
	EBV+		EBV-		EBV?		All cases		Controls		EBV+ versus EBV-				EBV+ versus controls				EBV- versus controls				all cases versus controls			
											OR	99.9%CI		p-value	OR	99.9%CI		p-value	OR	99.9%CI		p-value	OR	99.9%CI		p-value
DP04:02 /105:01	5	6.4	40	17.3	6	22.2	51	15.2			0.33	0.06	1.66	1.8E-02												
DP03:01	21	26.9	76	32.9	10	37.0	107	31.8			0.75	0.29	1.96	3.3E-01												
DP04:01	60	76.9	150	64.9	13	48.1	223	66.4			1.80	0.67	4.86	5.0E-02												
DP05:01	3	3.8	8	3.5	0	0.0	11	3.3			1.12	0.12	10.80	8.7E-01												
DP06:01	3	3.8	5	2.2	3	11.1	11	3.3			1.81	0.16	20.80	4.2E-01												
DP10:01	1	1.3	4	1.7	1	3.7	6	1.8			0.74	0.02	29.94	7.9E-01												
DP13:01	4	5.1	9	3.9	0	0.0	13	3.9			1.33	0.18	10.11	6.4E-01												
DP14:01	1	1.3	13	5.6	0	0.0	14	4.2			0.22	0.01	6.81	1.1E-01												
DP19:01	5	6.4	8	3.5	0	0.0	13	3.9			1.91	0.28	13.12	2.6E-01												
Others	3	3.9	24	10.4	3	11.1	30	8.9			0.35	0.04	2.73	7.7E-02												

* Allelic frequency < 1% was excluded

Table S3 Individual PCR-SSOP analysis including OR and p-values

GENE	PROBE	POS	EBV+	EBV-	NMISS	P	OR (99. 9% CI)
HLA-DPB1	c22	32	96, 2%	85, 8%	311	0, 011	5. 2 (0. 6-43. 1)
HLA-DPB1	c43	35	96, 2%	85, 8%	311	0, 011	5. 2 (0. 6-43. 1)
HLA-DPB1	c12	35	2, 6%	10, 3%	311	0, 026	0. 2 (0. 0-2. 3)
HLA-DPB1	c47	35	96, 2%	90, 1%	311	0, 034	4. 1 (0. 5-36. 0)
HLA-DPB1	c01	35	5, 1%	9, 0%	311	0, 56	0. 7 (0. 1-4. 9)
HLA-DPB1	c11	35	38, 5%	41, 2%	311	0, 67	0. 9 (0. 3-2. 3)
HLA-DPB1	c45	35	0, 0%	0, 0%	311	n. a.	n. a. (n. a. -n. a.)
HLA-DPB1	c23	49	0, 0%	0, 0%	309	n. a.	n. a. (n. a. -n. a.)
HLA-DPB1	c13	105	1, 3%	3, 0%	311	0, 44	0. 4 (0. 0-17. 0)
HLA-DPB1	c63	105	5, 1%	4, 3%	311	0, 79	0. 8 (0. 1-7. 0)
HLA-DPB1	c64	105	0, 0%	0, 4%	309	1, 00	n. a. (n. a. -n. a.)
HLA-DPB1	c09	107	76, 9%	60, 5%	311	8, 8E-03	2. 3 (0. 8-6. 6)
HLA-DPB1	c50	107	76, 9%	60, 5%	311	8, 8E-03	2. 3 (0. 8-6. 6)
HLA-DPB1	c31	107	65, 4%	78, 1%	311	0, 022	0. 5 (0. 2-1. 4)
HLA-DPB1	c56	107	38, 2%	48, 1%	278	0, 13	0. 6 (0. 2-1. 7)
HLA-DPB1	c10	107	5, 1%	3, 0%	311	0, 42	1. 7 (0. 2-16. 7)
HLA-DPB1	c54	107	44, 4%	50, 7%	291	0, 51	0. 8 (0. 3-2. 2)
HLA-DPB1	c52	107	5, 1%	8, 6%	311	0, 60	0. 7 (0. 1-5. 1)
HLA-DPB1	c02	107	10, 3%	12, 5%	311	0, 63	0. 8 (0. 2-3. 5)
HLA-DPB1	c66	107	1, 3%	0, 0%	311	1, 00	n. a. (n. a. -n. a.)
HLA-DPB1	c51	127	5, 1%	3, 0%	311	0, 42	1. 7 (0. 2-16. 7)
HLA-DPB1	c14	129	1, 3%	2, 2%	309	0, 95	0. 9 (0. 0-41. 1)
HLA-DPB1	c59	171	1, 3%	8, 6%	309	0, 043	0. 1 (0. 0-3. 8)
HLA-DPB1	c08	171	82, 1%	71, 2%	311	0, 044	2. 0 (0. 6-6. 4)
HLA-DPB1	c05	171	37, 2%	45, 9%	311	0, 17	0. 7 (0. 3-1. 7)
HLA-DPB1	c57	171	33, 3%	42, 2%	310	0, 18	0. 7 (0. 3-1. 8)
HLA-DPB1	c07	171	33, 3%	42, 1%	311	0, 20	0. 7 (0. 3-1. 8)
HLA-DPB1	c17	171	11, 5%	6, 4%	311	0, 20	1. 8 (0. 4-8. 9)

continued

GENE	PROBE	POS	EBV+	EBV-	NMISS	P	OR (99. 9% CI)
HLA-DPB1	c62	171	1, 3%	1, 8%	296	0, 51	0. 4 (0. 0-24. 6)
HLA-DPB1	c67	171	1, 3%	0, 0%	304	1, 00	n. a. (n. a. -n. a.)
HLA-DPB1	c19	171	0, 0%	0, 0%	311	n. a.	n. a. (n. a. -n. a.)
HLA-DPB1	c44	199	98, 7%	92, 7%	311	0, 073	6. 7 (0. 2-223. 8)
HLA-DPB1	c27	199	34, 6%	42, 1%	311	0, 32	0. 7 (0. 3-2. 0)
HLA-DPB1	c21	199	0, 0%	0, 0%	309	n. a.	n. a. (n. a. -n. a.)
HLA-DPB1	c24	199	0, 0%	0, 0%	311	n. a.	n. a. (n. a. -n. a.)
HLA-DPB1	c70	206	3, 8%	2, 1%	311	0, 64	1. 4 (0. 1-19. 7)
HLA-DPB1	c71	208	7, 7%	25, 3%	311	3, 2E-03	0. 3 (0. 1-1. 2)
HLA-DPB1	c60	208	1, 3%	4, 7%	311	0, 10	0. 2 (0. 0-6. 1)
HLA-DPB1	c04	208	43, 6%	30, 9%	311	0, 17	1. 5 (0. 6-3. 9)
HLA-DPB1	c06	208	29, 5%	38, 2%	311	0, 25	0. 7 (0. 3-1. 9)
HLA-DPB1	c58	208	28, 2%	34, 2%	309	0, 46	0. 8 (0. 3-2. 2)
HLA-DPB1	c25	208	0, 0%	0, 0%	311	n. a.	n. a. (n. a. -n. a.)
HLA-DPB1	c72	210	0, 0%	0, 0%	311	n. a.	n. a. (n. a. -n. a.)
HLA-DPB1	c30	216	93, 6%	93, 1%	311	0, 48	1. 5 (0. 2-9. 7)
HLA-DPB1	c20	218	0, 0%	0, 0%	311	n. a.	n. a. (n. a. -n. a.)
HLA-DPB1	c40	226	0, 0%	0, 0%	311	n. a.	n. a. (n. a. -n. a.)
HLA-DPB1	c41	228	98, 7%	90, 0%	304	0, 047	8. 0 (0. 3-252. 8)
HLA-DPB1	c29	228	34, 6%	45, 1%	311	0, 20	0. 7 (0. 3-1. 8)
HLA-DPB1	c53	228	5, 1%	8, 6%	311	0, 60	0. 7 (0. 1-5. 1)
HLA-DPB1	c15	228	11, 5%	7, 7%	311	0, 64	1. 2 (0. 3-5. 7)
HLA-DPB1	c69	228	6, 5%	6, 0%	309	0, 75	0. 8 (0. 1-5. 5)
HLA-DPB1	c65	228	0, 0%	2, 2%	309	1, 00	n. a. (n. a. -n. a.)
HLA-DPB1	c61	256	2, 6%	10, 3%	311	0, 026	0. 2 (0. 0-2. 3)
HLA-DPB1	c03	256	91, 0%	82, 4%	305	0, 041	2. 6 (0. 6-11.6mm)
HLA-DPB1	c42	256	50, 0%	59, 9%	310	0, 14	0. 7 (0. 3-1. 7)
HLA-DPB1	c16	259	0, 0%	0, 0%	310	n. a.	n. a. (n. a. -n. a.)

continued

GENE	PROBE	POS	EBV+	EBV-	NMISS	P	OR (99. 9% CI)
HLA-DQB1	c257	26	93, 3%	93, 8%	302	0, 42	0. 6 (0. 1-4. 3)
HLA-DQB1	c251	40	18, 4%	35, 8%	308	8, 6E-03	0. 4 (0. 1-1. 3)
HLA-DQB1	c240	40	32, 9%	25, 4%	308	0, 56	1. 2 (0. 4-3. 4)
HLA-DQB1	c250	40	32, 9%	25, 4%	308	0, 56	1. 2 (0. 4-3. 4)
HLA-DQB1	c216	75	8, 1%	2, 6%	305	0, 16	2. 5 (0. 3-21. 3)
HLA-DQB1	c241	77	0, 0%	0, 0%	308	n. a.	n. a. (n. a. -n. a.)
HLA-DQB1	c272	79	0, 0%	0, 0%	305	n. a.	n. a. (n. a. -n. a.)
HLA-DQB1	c226	83	38, 4%	28, 1%	304	0, 45	1. 3 (0. 5-3. 5)
HLA-DQB1	c255	83	50, 0%	53, 9%	306	0, 72	0. 9 (0. 4-2. 3)
HLA-DQB1	c205	89	18, 4%	35, 9%	307	8, 6E-03	0. 4 (0. 1-1. 3)
HLA-DQB1	c232	89	8, 1%	3, 0%	305	0, 16	2. 5 (0. 3-21. 3)
HLA-DQB1	c201	90	32, 9%	25, 4%	308	0, 56	1. 2 (0. 4-3. 4)
HLA-DQB1	c212	90	32, 9%	28, 9%	308	0, 61	1. 2 (0. 4-3. 2)
HLA-DQB1	c207	90	50, 0%	53, 9%	308	0, 70	0. 9 (0. 4-2. 3)
HLA-DQB1	c206	90	27, 6%	25, 0%	308	0, 94	1. 0 (0. 4-2. 9)
HLA-DQB1	c211	90	0, 0%	0, 4%	308	1, 00	n. a. (n. a. -n. a.)
HLA-DQB1	c221	111	0, 0%	0, 9%	295	1, 00	n. a. (n. a. -n. a.)
HLA-DQB1	c270	114	29, 0%	18, 6%	307	0, 082	1. 8 (0. 6-5. 2)
HLA-DQB1	c242	114	42, 1%	48, 3%	308	0, 37	0. 8 (0. 3-2. 0)
HLA-DQB1	c256	114	55, 3%	58, 6%	308	0, 60	0. 9 (0. 3-2. 2)
HLA-DQB1	c233	114	1, 3%	0, 0%	307	1, 00	n. a. (n. a. -n. a.)
HLA-DQB1	c213	135	17, 1%	35, 3%	308	6, 9E-03	0. 4 (0. 1-1. 2)
HLA-DQB1	c223	144	42, 1%	53, 9%	308	0, 11	0. 6 (0. 2-1.6mm)
HLA-DQB1	c202	147	26, 3%	19, 4%	308	0, 69	1. 1 (0. 4-3. 5)
HLA-DQB1	c263	147	0, 0%	0, 0%	308	n. a.	n. a. (n. a. -n. a.)
HLA-DQB1	c235	153	25, 7%	40, 6%	298	0, 039	0. 5 (0. 2-1. 5)
HLA-DQB1	c217	168	10, 5%	3, 0%	308	0, 031	3. 5 (0. 5-24. 1)
HLA-DQB1	c253	171	17, 1%	34, 9%	308	7, 5E-03	0. 4 (0. 1-1. 2)

continued

GENE	PROBE	POS	EBV+	EBV-	NMISS	P	OR (99. 9% CI)
HLA-DQB1	c214	171	20, 3%	36, 6%	290	9, 8E-03	0. 4 (0. 1-1. 3)
HLA-DQB1	c215	171	24, 0%	12, 2%	305	0, 019	2. 4 (0. 7-7. 8)
HLA-DQB1	c203	171	2, 6%	1, 7%	308	0, 30	2. 7 (0. 1-65. 0)
HLA-DQB1	c243	171	32, 0%	26, 3%	299	0, 50	1. 2 (0. 4-3. 4)
HLA-DQB1	c262	171	32, 9%	28, 3%	306	0, 55	1. 2 (0. 4-3. 3)
HLA-DQB1	c244	171	0, 0%	0, 0%	308	n. a.	n. a. (n. a. -n. a.)
HLA-DQB1	c204	178	31, 6%	24, 1%	308	0, 59	1. 2 (0. 4-3. 4)
HLA-DQB1	c208	180	42, 1%	53, 9%	308	0, 11	0. 6 (0. 2-1.6mm)
HLA-DQB1	c265	189	0, 0%	0, 0%	308	n. a.	n. a. (n. a. -n. a.)
HLA-DQB1	c252	200	93, 4%	96, 6%	308	0, 36	0. 6 (0. 1-4. 5)
HLA-DQB1	c264	200	0, 0%	0, 0%	290	n. a.	n. a. (n. a. -n. a.)
HLA-DQB1	c273	213	43, 2%	54, 8%	302	0, 15	0. 7 (0. 3-1. 7)
HLA-DQB1	c271	213	42, 7%	53, 7%	304	0, 17	0. 7 (0. 3-1. 7)
HLA-DQB1	c245	213	32, 9%	25, 4%	308	0, 56	1. 2 (0. 4-3. 4)
HLA-DQB1	c237	216	9, 3%	3, 4%	307	0, 089	2. 8 (0. 4-20. 0)
HLA-DQB1	c236	216	48, 0%	50, 9%	303	0, 58	0. 9 (0. 3-2. 2)
HLA-DQB1	c209	216	1, 3%	0, 4%	308	0, 78	1. 5 (0. 0-191. 0)
HLA-DQB1	c303	405	2, 6%	5, 6%	307	0, 18	0. 3 (0. 0-5. 0)
HLA-DQB1	c304	420	31, 6%	24, 2%	307	0, 30	1. 4 (0. 5-3. 9)
HLA-DQB1	c301	504	0, 0%	0, 0%	306	n. a.	n. a. (n. a. -n. a.)
HLA-DRB1	C17	25	37, 2%	50, 7%	303	0, 037	0. 5 (0. 2-1. 4)
HLA-DRB1	C12	25	7, 7%	2, 2%	303	0, 68	1. 3 (0. 1-12. 6)
HLA-DRB1	C04	31	29, 5%	18, 2%	303	0, 024	2. 1 (0. 7-6. 1)
HLA-DRB1	C73	31	9, 0%	11, 1%	303	0, 32	0. 6 (0. 1-3. 0)
HLA-DRB1	C11	31	0, 0%	2, 2%	303	1, 00	n. a. (n. a. -n. a.)
HLA-DRB1	C47	32	20, 5%	17, 8%	303	0, 73	1. 1 (0. 3-3. 7)
HLA-DRB1	C10	36	9, 0%	10, 9%	307	0, 33	0. 6 (0. 1-3. 1)
HLA-DRB1	C02	36	32, 1%	40, 0%	303	0, 35	0. 8 (0. 3-2. 0)

continued

GENE	PROBE	POS	EBV+	EBV-	NMISS	P	OR (99. 9% CI)
HLA-DRB1	C18	37	32, 1%	40, 0%	303	0, 35	0. 8 (0. 3-2. 0)
HLA-DRB1	C08	39	12, 8%	6, 1%	307	0, 12	2. 1 (0. 4-9. 6)
HLA-DRB1	C07	39	66, 7%	67, 3%	307	0, 97	1. 0 (0. 4-2. 7)
HLA-DRB1	C34	76	32, 1%	46, 9%	302	0, 024	0. 5 (0. 2-1. 4)
HLA-DRB1	C20	76	35, 9%	29, 5%	302	0, 36	1. 3 (0. 5-3. 5)
HLA-DRB1	C39	86	38, 2%	31, 1%	295	0, 30	1. 4 (0. 5-3. 7)
HLA-DRB1	C63	88	28, 2%	39, 1%	303	0, 16	0. 6 (0. 2-1. 8)
HLA-DRB1	C01	88	20, 5%	17, 9%	307	0, 77	1. 1 (0. 3-3. 6)
HLA-DRB1	C33	90	0, 0%	0, 0%	307	n. a.	n. a. (n. a. -n. a.)
HLA-DRB1	C27	94	7, 7%	5, 2%	307	0, 37	1. 7 (0. 2-11. 3)
HLA-DRB1	C84	94	7, 7%	2, 2%	307	0, 66	1. 3 (0. 1-12. 8)
HLA-DRB1	C49	111	3, 8%	3, 1%	307	0, 76	1. 3 (0. 1-14. 2)
HLA-DRB1	C06	111	2, 6%	2, 6%	307	1, 00	1. 0 (0. 1-17. 5)
HLA-DRB1	C35	112	28, 2%	39, 1%	303	0, 16	0. 6 (0. 2-1. 8)
HLA-DRB1	C36	112	23, 1%	21, 8%	307	0, 86	1. 1 (0. 3-3. 3)
HLA-DRB1	C25	113	53, 9%	45, 4%	307	0, 44	1. 2 (0. 5-3. 1)
HLA-DRB1	C94	113	8, 6%	4, 5%	235	0, 50	1. 5 (0. 2-12. 4)
HLA-DRB1	C16	115	38, 5%	28, 9%	303	0, 090	1. 7 (0. 6-4. 4)
HLA-DRB1	C72	150	0, 0%	0, 0%	307	n. a.	n. a. (n. a. -n. a.)
HLA-DRB1	C74	176	9, 0%	24, 0%	307	0, 011	0. 3 (0. 1-1. 4)
HLA-DRB1	C05	178	9, 0%	24, 0%	307	0, 011	0. 3 (0. 1-1. 4)
HLA-DRB1	C23	178	7, 7%	7, 0%	307	0, 51	1. 4 (0. 2-8. 2)
HLA-DRB1	C29	179	11, 5%	4, 0%	303	0, 043	3. 0 (0. 5-17. 3)
HLA-DRB1	C19	179	50, 0%	46, 2%	303	0, 78	1. 1 (0. 4-2. 7)
HLA-DRB1	C79	202	0, 0%	0, 0%	301	n. a.	n. a. (n. a. -n. a.)
HLA-DRB1	C40	208	30, 8%	30, 1%	307	0, 92	1. 0 (0. 4-2. 7)
HLA-DRB1	C37	211	0, 0%	0, 0%	303	n. a.	n. a. (n. a. -n. a.)
HLA-DRB1	C48	212	29, 5%	39, 3%	302	0, 21	0. 7 (0. 3-1. 8)

continued

GENE	PROBE	POS	EBV+	EBV-	NMISS	P	OR (99. 9% CI)
HLA-DRB1	C86	212	29, 5%	38, 8%	302	0, 22	0. 7 (0. 3-1. 9)
HLA-DRB1	C14	213	32, 1%	39, 7%	307	0, 34	0. 8 (0. 3-2. 0)
HLA-DRB1	C13	213	29, 5%	27, 1%	307	0, 87	1. 1 (0. 4-2. 9)
HLA-DRB1	C24	213	0, 0%	1, 3%	307	1, 00	n. a. (n. a. -n. a.)
HLA-DRB1	C85	214	2, 6%	0, 9%	303	0, 26	3. 3 (0. 1-102. 4)
HLA-DRB1	C53	215	0, 0%	0, 0%	307	n. a.	n. a. (n. a. -n. a.)
HLA-DRB1	C21	216	39, 7%	22, 7%	307	0, 017	2. 1 (0. 8-5. 6)
HLA-DRB1	C80	216	2, 6%	0, 9%	303	0, 26	3. 3 (0. 1-102. 4)
HLA-DRB1	C41	216	18, 0%	23, 1%	307	0, 47	0. 8 (0. 2-2. 5)
HLA-DRB1	C64	219	29, 5%	29, 3%	307	0, 82	0. 9 (0. 3-2. 6)
HLA-DRB1	C51	221	9, 0%	20, 5%	307	0, 049	0. 4 (0. 1-1. 8)
HLA-DRB1	C38	221	9, 1%	11, 2%	300	0, 84	1. 1 (0. 2-5. 3)
HLA-DRB1	C43	222	34, 6%	19, 2%	302	0, 022	2. 1 (0. 7-5. 8)
HLA-DRB1	C09	222	10, 3%	3, 5%	307	0, 068	2. 8 (0. 4-17. 1)
HLA-DRB1	C15	222	43, 6%	35, 1%	306	0, 16	1. 5 (0. 6-3. 9)
HLA-DRB1	C67	229	10, 3%	10, 7%	303	0, 84	1. 1 (0. 2-5. 0)
HLA-DRB1	C03	231	28, 2%	23, 6%	307	0, 47	1. 3 (0. 4-3. 6)
HLA-DRB1	C26	255	5, 1%	4, 4%	307	0, 81	1. 2 (0. 1-9. 7)
HLA-DRB1	C87	258	56, 4%	61, 6%	302	0, 50	0. 8 (0. 3-2. 1)
HLA-DRB1	C89	259	1, 3%	0, 4%	303	0, 56	2. 6 (0. 0-587. 1)
HLA-DRB3	C32mix	48	0, 0%	0, 0%	205	n. a.	n. a. (n. a. -n. a.)
HLA-DRB3	C39mix	86	40, 0%	32, 4%	198	0, 49	1. 3 (0. 4-4. 1)
HLA-DRB3	C57mix	90	68, 6%	77, 9%	205	0, 26	0. 6 (0. 2-2. 3)
HLA-DRB3	C27mix	94	0, 0%	0, 0%	205	n. a.	n. a. (n. a. -n. a.)
HLA-DRB3	C36mix	112	11, 8%	10, 4%	205	0, 42	1.6mm (0. 3-9. 4)
HLA-DRB3	C25mix	113	80, 4%	67, 3%	204	0, 27	1.6mm (0. 4-6. 1)
HLA-DRB3	C45mix	117	13, 7%	37, 7%	205	4, 1E-03	0. 3 (0. 1-1. 2)
HLA-DRB3	C76mix	143	11, 8%	12, 3%	205	0, 63	1. 3 (0. 2-7. 5)

continued

GENE	PROBE	POS	EBV+	EBV-	NMISS	P	OR (99. 9% CI)
HLA-DRB3	C46mix	148	92, 2%	92, 1%	203	0, 54	0. 7 (0. 1-5. 6)
HLA-DRB3	C74mix	176	13, 7%	36, 4%	205	5, 9E-03	0. 3 (0. 1-1. 3)
HLA-DRB3	C78mix	177	0, 0%	0, 0%	205	n. a.	n. a. (n. a. -n. a.)
HLA-DRB3	C05mix	178	13, 7%	36, 4%	205	5, 9E-03	0. 3 (0. 1-1. 3)
HLA-DRB3	C23mix	178	11, 8%	10, 4%	205	0, 42	1.6mm (0. 3-9. 4)
HLA-DRB3	C44mix	179	80, 4%	66, 9%	205	0, 24	1.6mm (0. 4-6. 3)
HLA-DRB3	C29mix	179	0, 0%	1, 9%	205	1, 00	n. a. (n. a. -n. a.)
HLA-DRB3	C88mix	179	0, 0%	0, 0%	205	n. a.	n. a. (n. a. -n. a.)
HLA-DRB3	C28mix	199	13, 7%	31, 2%	205	0, 032	0. 4 (0. 1-1. 7)
HLA-DRB3	C69mix	208	0, 0%	0, 0%	205	n. a.	n. a. (n. a. -n. a.)
HLA-DRB3	C97mix	212	0, 0%	5, 2%	205	1, 00	n. a. (n. a. -n. a.)
HLA-DRB3	C13mix	213	45, 1%	37, 7%	205	0, 47	1. 3 (0. 4-4. 0)
HLA-DRB3	C14mix	213	0, 0%	0, 0%	205	n. a.	n. a. (n. a. -n. a.)
HLA-DRB3	C98mix	216	13, 7%	31, 2%	205	0, 032	0. 4 (0. 1-1. 7)
HLA-DRB3	C21mix	216	0, 0%	0, 0%	205	n. a.	n. a. (n. a. -n. a.)
HLA-DRB3	C51mix	221	13, 7%	31, 2%	205	0, 032	0. 4 (0. 1-1. 7)
HLA-DRB3	C15mix	222	11, 8%	10, 4%	205	0, 42	1.6mm (0. 3-9. 4)
HLA-DRB3	C70mix	222	41, 2%	35, 1%	205	0, 55	1. 2 (0. 4-3. 9)
HLA-DRB3	C93mix	230	0, 0%	0, 0%	205	n. a.	n. a. (n. a. -n. a.)
HLA-DRB3	C30mix	250	86, 3%	81, 1%	204	0, 39	1. 5 (0. 3-7. 3)
HLA-DRB3	C31mix	258	21, 6%	26, 1%	204	0, 75	0. 9 (0. 2-3. 4)
HLA-B	c282	98	28, 4%	23, 1%	295	0, 44	1. 3 (0. 4-3. 8)
HLA-B	c261	106	34, 2%	40, 2%	305	0, 31	0. 7 (0. 3-2. 0)
HLA-B	c265	106	2, 6%	3, 1%	307	0, 85	1. 2 (0. 1-21. 1)
HLA-B	c268	141	15, 6%	13, 2%	305	0, 66	1. 2 (0. 3-4. 3)
HLA-B	c224	146	38, 9%	45, 2%	280	0, 25	0. 7 (0. 3-1. 9)
HLA-B	c287	167	41, 6%	36, 1%	304	0, 43	1. 3 (0. 5-3. 3)
HLA-B	c213	207	62, 8%	66, 4%	307	0, 50	0. 8 (0. 3-2. 1)

continued

GENE	PROBE	POS	EBV+	EBV-	NMISS	P	OR (99. 9% CI)
HLA-B	c212	210	21, 8%	24, 9%	307	0, 57	0. 8 (0. 3-2. 5)
HLA-B	c210	213	35, 9%	31, 0%	307	0, 56	1. 2 (0. 4-3. 2)
HLA-B	c211	215	51, 3%	41, 0%	305	0, 42	1. 3 (0. 5-3. 2)
HLA-B	c226	250	6, 4%	8, 3%	307	0, 47	0. 7 (0. 1-4. 2)
HLA-B	c264	258	9, 0%	3, 1%	307	0, 14	2. 4 (0. 3-16. 7)
HLA-B	c234	269	59, 0%	53, 7%	307	0, 93	1. 0 (0. 4-2. 6)
HLA-B	c266	270	85, 9%	82, 5%	307	0, 59	1. 2 (0. 3-4. 5)
HLA-B	c203	273	9, 0%	3, 5%	307	0, 21	2. 0 (0. 3-13. 3)
HLA-B	c207	277	7, 7%	8, 7%	307	0, 60	0. 8 (0. 1-4. 2)
HLA-B	c202	277	0, 0%	0, 0%	307	n. a.	n. a. (n. a. -n. a.)
HLA-B	c204	278	62, 8%	56, 6%	306	0, 75	1. 1 (0. 4-2. 8)
HLA-B	c206	279	55, 1%	52, 4%	307	0, 76	1. 1 (0. 4-2. 8)
HLA-B	c205	280	30, 8%	36, 2%	307	0, 47	0. 8 (0. 3-2. 2)
HLA-B	c235	280	30, 8%	36, 2%	307	0, 47	0. 8 (0. 3-2. 2)
HLA-B	c292	280	0, 0%	0, 0%	307	n. a.	n. a. (n. a. -n. a.)
HLA-B	c201	282	1, 3%	7, 0%	307	0, 22	0. 3 (0. 0-8. 9)
HLA-B	c285	283	30, 8%	36, 2%	307	0, 47	0. 8 (0. 3-2. 2)
HLA-B	c233	283	0, 0%	0, 0%	307	n. a.	n. a. (n. a. -n. a.)
HLA-B	c246	302	0, 0%	0, 0%	307	n. a.	n. a. (n. a. -n. a.)
HLA-B	c274	303	1, 3%	7, 0%	307	0, 22	0. 3 (0. 0-8. 9)
HLA-B	c216	303	0, 0%	0, 4%	307	1, 00	n. a. (n. a. -n. a.)
HLA-B	c218	304	44, 9%	54, 0%	306	0, 17	0. 7 (0. 3-1. 7)
HLA-B	c215	304	39, 7%	38, 9%	307	0, 53	0. 8 (0. 3-2. 2)
HLA-B	c277	314	88, 5%	93, 5%	307	0, 18	0. 5 (0. 1-2. 6)
HLA-B	c220	314	14, 1%	18, 3%	307	0, 23	0. 6 (0. 2-2. 3)
HLA-B	c278	314	0, 0%	1, 7%	307	1, 00	n. a. (n. a. -n. a.)
HLA-B	c222	319	18, 0%	11, 4%	307	0, 55	1. 3 (0. 3-4. 8)
HLA-B	c231	320	19, 2%	15, 1%	303	0, 50	1. 3 (0. 4-4. 3)

continued

GENE	PROBE	POS	EBV+	EBV-	NMISS	P	OR (99. 9% CI)
HLA-B	c291	320	15, 6%	13, 2%	305	0, 66	1. 2 (0. 3-4. 3)
HLA-B	c244	320	16, 7%	14, 4%	307	0, 69	1. 2 (0. 3-4. 1)
HLA-B	c229	320	15, 4%	13, 5%	307	0, 78	1. 1 (0. 3-4. 1)
HLA-B	c232	320	0, 0%	1, 7%	307	1, 00	n. a. (n. a. -n. a.)
HLA-B	c283	322	51, 3%	48, 3%	306	0, 47	0. 8 (0. 3-2. 1)
HLA-B	c286	322	11, 7%	10, 6%	304	0, 75	1. 2 (0. 3-5. 0)
HLA-B	c221	324	88, 5%	93, 5%	307	0, 18	0. 5 (0. 1-2. 6)
HLA-B	c223	324	26, 9%	22, 3%	307	0, 78	1. 1 (0. 4-3. 2)
HLA-B	c332	363	9, 0%	3, 1%	307	0, 14	2. 4 (0. 3-16. 7)
HLA-B	c327	363	29, 5%	34, 8%	305	0, 31	0. 7 (0. 3-2. 0)
HLA-B	c331	363	3, 8%	3, 9%	307	0, 84	1. 2 (0. 1-13. 2)
HLA-B	c319	369	1, 3%	6, 6%	306	0, 27	0. 3 (0. 0-10. 1)
HLA-B	c330	369	6, 4%	5, 3%	306	0, 35	1. 8 (0. 2-13. 0)
HLA-B	c333	369	42, 3%	46, 9%	304	0, 51	0. 8 (0. 3-2. 1)
HLA-B	c324	369	20, 5%	19, 2%	307	0, 74	1. 1 (0. 4-3. 5)
HLA-B	c334	369	59, 7%	57, 5%	305	0, 82	1. 1 (0. 4-2. 8)
HLA-B	c329	369	0, 0%	0, 0%	307	n. a.	n. a. (n. a. -n. a.)
HLA-B	c394	379	85, 9%	86, 8%	305	0, 96	1. 0 (0. 3-3. 8)
HLA-B	c339	404	0, 0%	2, 2%	307	1, 00	n. a. (n. a. -n. a.)
HLA-B	c336	414	62, 3%	56, 2%	303	0, 44	1. 3 (0. 5-3. 3)
HLA-B	c350	420	1, 3%	7, 4%	307	0, 18	0. 2 (0. 0-7. 8)
HLA-B	c340	420	2, 6%	0, 4%	306	0, 19	5. 1 (0. 1-304. 4)
HLA-B	c341	420	24, 4%	12, 3%	305	0, 22	1.6mm (0. 5-5. 2)
HLA-B	c311	420	57, 9%	47, 5%	297	0, 22	1. 4 (0. 6-3. 7)
HLA-B	c370	420	37, 7%	29, 7%	303	0, 30	1. 4 (0. 5-3. 7)
HLA-B	c355	420	5, 1%	4, 4%	307	0, 41	1. 8 (0. 2-16. 5)
HLA-B	c337	420	13, 0%	15, 4%	305	0, 41	0. 7 (0. 2-2. 8)
HLA-B	c335	420	37, 2%	41, 4%	305	0, 50	0. 8 (0. 3-2. 1)

continued

GENE	PROBE	POS	EBV+	EBV-	NMISS	P	OR (99. 9% CI)
HLA-B	c338	420	29, 9%	34, 2%	305	0, 57	0. 8 (0. 3-2. 3)
HLA-B	c389	420	1, 3%	1, 3%	307	0, 77	1. 4 (0. 0-74. 0)
HLA-B	c353	420	0, 0%	4, 8%	307	1, 00	n. a. (n. a. -n. a.)
HLA-B	c356	425	3, 8%	4, 8%	307	0, 50	0. 6 (0. 1-6. 2)
HLA-B	c393	512	10, 3%	7, 0%	307	0, 48	1. 4 (0. 3-7. 1)
HLA-B	c442	528	61, 5%	67, 7%	307	0, 66	0. 9 (0. 3-2. 3)
HLA-B	c459	528	83, 3%	81, 1%	306	0, 92	1. 0 (0. 3-3. 3)
HLA-B	c457	546	10, 4%	18, 9%	305	0, 12	0. 5 (0. 1-2. 1)
HLA-B	c443	546	51, 3%	42, 4%	307	0, 40	1. 3 (0. 5-3. 2)
HLA-B	c444	546	30, 8%	35, 4%	307	0, 57	0. 8 (0. 3-2. 3)
HLA-B	c416	548	3, 8%	4, 8%	307	0, 50	0. 6 (0. 1-6. 2)
HLA-B	c451	548	61, 5%	63, 3%	307	0, 97	1. 0 (0. 4-2. 6)
HLA-B	c478	560	11, 5%	18, 8%	307	0, 21	0. 6 (0. 2-2. 3)
HLA-B	c405	560	10, 3%	15, 3%	307	0, 24	0. 6 (0. 1-2. 5)
HLA-B	c325	560	16, 7%	13, 7%	305	0, 59	1. 2 (0. 3-4. 3)
HLA-B	c445	564	56, 4%	45, 4%	307	0, 15	1. 5 (0. 6-3. 8)
HLA-B	c462	566	62, 8%	62, 0%	307	0, 74	0. 9 (0. 3-2. 4)
HLA-B	c401	571	44, 9%	51, 1%	307	0, 44	0. 8 (0. 3-2. 0)
HLA-B	c448	573	14, 1%	17, 0%	307	0, 37	0. 7 (0. 2-2. 6)
HLA-B	c460	573	0, 0%	0, 0%	307	n. a.	n. a. (n. a. -n. a.)
HLA-B	c488	583	16, 9%	14, 4%	306	0, 66	1. 2 (0. 3-4. 1)
HLA-B	c447	584	26, 9%	25, 8%	307	0, 69	1. 1 (0. 4-3. 2)
HLA-B	c471	589	96, 2%	98, 7%	307	0, 13	0. 3 (0. 0-4. 9)
HLA-B	c417	614	82, 1%	80, 8%	307	0, 70	0. 9 (0. 3-2. 9)
HLA-B	c477	616	33, 3%	26, 8%	306	0, 28	1. 4 (0. 5-3. 8)
HLA-B	c494	618	39, 7%	40, 6%	307	0, 92	1. 0 (0. 4-2. 5)
HLA-C	c201	102	0, 0%	4, 6%	265	1, 00	n. a. (n. a. -n. a.)
HLA-C	c251	103	22, 1%	24, 7%	304	0, 56	0. 8 (0. 3-2. 5)

continued

GENE	PROBE	POS	EBV+	EBV-	NMISS	P	OR (99. 9% CI)
HLA-C	c237	108	22, 4%	24, 9%	301	0, 78	0. 9 (0. 3-2. 7)
HLA-C	c250	113	42, 9%	39, 8%	303	0, 35	1. 3 (0. 5-3. 4)
HLA-C	c253	121	7, 8%	10, 6%	304	0, 45	0. 7 (0. 1-3. 6)
HLA-C	c252	134	88, 3%	81, 9%	304	0, 44	1. 4 (0. 3-5. 5)
HLA-C	c243	134	24, 7%	27, 0%	303	0, 95	1. 0 (0. 3-2. 9)
HLA-C	c241	141	44, 0%	40, 0%	300	0, 29	1. 4 (0. 5-3. 6)
HLA-C	c249	142	75, 0%	67, 7%	302	0, 57	1. 2 (0. 4-3. 5)
HLA-C	c256	142	75, 3%	73, 5%	303	0, 82	0. 9 (0. 3-2. 7)
HLA-C	c255	144	44, 2%	46, 7%	304	0, 69	0. 9 (0. 3-2. 3)
HLA-C	c246	181	16, 9%	17, 2%	304	0, 69	1. 2 (0. 3-4. 0)
HLA-C	c266	270	90, 9%	96, 4%	300	0, 035	0. 3 (0. 0-2. 0)
HLA-C	c228	272	40, 3%	36, 1%	304	0, 41	1. 3 (0. 5-3. 3)
HLA-C	c267	296	59, 7%	57, 7%	304	0, 31	1. 3 (0. 5-3. 6)
HLA-C	c247	312	81, 8%	88, 1%	304	0, 23	0. 6 (0. 2-2. 3)
HLA-C	c271	312	9, 2%	12, 4%	302	0, 61	0. 8 (0. 2-3. 7)
HLA-C	c268	312	63, 9%	62, 4%	290	0, 83	1. 1 (0. 4-2. 9)
HLA-C	c231	312	63, 6%	56, 8%	304	0, 93	1. 0 (0. 4-2. 6)
HLA-C	c224	343	3, 9%	13, 2%	303	0, 072	0. 3 (0. 0-2. 6)
HLA-C	c272	343	84, 0%	81, 1%	292	0, 69	1. 2 (0. 3-4. 1)
HLA-C	c335	368	35, 3%	17, 9%	258	0, 092	1. 8 (0. 6-5. 6)
HLA-C	c313	368	29, 7%	34, 8%	295	0, 57	0. 8 (0. 3-2. 3)
HLA-C	c318	368	2, 6%	3, 5%	302	0, 83	1. 2 (0. 1-18. 7)
HLA-C	c332	369	9, 1%	10, 2%	302	0, 62	0. 8 (0. 2-3. 9)
HLA-C	c306	387	19, 5%	22, 0%	304	0, 45	0. 8 (0. 2-2. 5)
HLA-C	c319	411	10, 4%	5, 3%	304	0, 11	2. 3 (0. 4-12. 5)
HLA-C	c369	419	37, 7%	39, 7%	304	0, 86	0. 9 (0. 4-2. 5)
HLA-C	c359	420	9, 1%	4, 8%	304	0, 10	2. 4 (0. 4-14. 4)
HLA-C	c336	420	39, 0%	42, 0%	301	0, 68	0. 9 (0. 3-2. 3)

continued

GENE	PROBE	POS	EBV+	EBV-	NMISS	P	OR (99. 9% CI)
HLA-C	c358	420	1, 3%	0, 0%	304	1, 00	n. a. (n. a. -n. a.)
HLA-C	c302	421	20, 3%	30, 2%	296	0, 17	0. 6 (0. 2-1. 9)
HLA-C	c330	427	87, 0%	80, 2%	299	0, 34	1. 5 (0. 4-5. 4)
HLA-C	c309	456	16, 9%	17, 2%	304	0, 69	1. 2 (0. 3-4. 0)
HLA-C	c316	481	1, 3%	0, 9%	304	0, 93	1. 1 (0. 0-97. 8)
HLA-C	c323	481	1, 3%	1, 8%	304	0, 97	1. 1 (0. 0-52. 4)
HLA-C	c361	486	6, 6%	8, 1%	298	0, 63	0. 8 (0. 1-4. 7)
HLA-C	c312	512	59, 7%	61, 2%	304	0, 80	0. 9 (0. 4-2. 4)
HLA-C	c372	527	1, 3%	1, 3%	303	0, 75	1. 5 (0. 0-77. 7)
HLA-C	c325	527	61, 0%	61, 5%	303	0, 76	0. 9 (0. 4-2. 4)
HLA-C	c368	527	59, 7%	61, 1%	303	0, 78	0. 9 (0. 4-2. 4)
HLA-C	c370	531	80, 0%	77, 8%	296	0, 77	0. 9 (0. 3-2. 9)
HLA-C	c357	540	39, 0%	25, 1%	304	0, 34	1. 3 (0. 5-3. 7)
HLA-C	c338	543	35, 5%	44, 3%	302	0, 25	0. 7 (0. 3-1. 9)
HLA-C	c314	543	1, 3%	2, 6%	304	0, 27	0. 3 (0. 0-11. 8)
HLA-C	c320	544	3, 9%	1, 3%	303	0, 40	2. 1 (0. 1-39. 0)
HLA-C	c339	544	78, 7%	80, 1%	301	0, 79	1. 1 (0. 3-3. 5)
HLA-C	c304	559	24, 7%	27, 3%	304	0, 90	1. 0 (0. 3-2. 8)
HLA-C	c303	564	9, 1%	12, 0%	303	0, 56	0. 8 (0. 2-3. 6)
HLA-C	c321	583	1, 3%	1, 8%	304	0, 97	1. 1 (0. 0-52. 4)
HLA-C	c362	598	24, 7%	27, 3%	304	0, 90	1. 0 (0. 3-2. 8)
HLA-C	c360	601	18, 2%	19, 4%	304	0, 95	1. 0 (0. 3-3. 2)
HLA-C	c327	601	0, 0%	0, 0%	304	n. a.	n. a. (n. a. -n. a.)
HLA-A	c204	102	19, 5%	18, 3%	306	0, 93	1. 0 (0. 3-3. 4)
HLA-A	c202	108	23, 1%	20, 2%	306	0, 40	1. 3 (0. 4-4. 0)
HLA-A	c205	108	0, 0%	1, 7%	310	1, 00	n. a. (n. a. -n. a.)
HLA-A	c234	123	1, 3%	0, 0%	310	1, 00	n. a. (n. a. -n. a.)
HLA-A	c206	129	0, 0%	0, 0%	310	n. a.	n. a. (n. a. -n. a.)

continued

GENE	PROBE	POS	EBV+	EBV-	NMISS	P	OR (99. 9% CI)
HLA-A	c278	144	2, 6%	0, 4%	310	0, 10	8. 0 (0. 1-548. 7)
HLA-A	c279	147	23, 0%	50, 0%	294	4, 3E-04	0. 3 (0. 1-0. 9)
HLA-A	c280	180	10, 3%	9, 1%	310	0, 88	1. 1 (0. 2-4. 9)
HLA-A	c281	203	55, 8%	29, 3%	306	1, 7E-04	3. 0 (1. 2-7. 9)
HLA-A	c266	203	87, 0%	93, 5%	307	0, 14	0. 5 (0. 1-2. 4)
HLA-A	c237	203	1, 3%	0, 0%	308	1, 00	n. a. (n. a. -n. a.)
HLA-A	c207	245	2, 6%	6, 9%	310	0, 31	0. 5 (0. 0-6. 0)
HLA-A	c210	261	2, 6%	3, 4%	310	0, 59	0. 6 (0. 0-9. 6)
HLA-A	c211	264	87, 2%	68, 0%	309	1, 6E-03	3. 4 (0. 9-12. 0)
HLA-A	c208	265	29, 5%	53, 0%	310	2, 9E-03	0. 4 (0. 2-1. 1)
HLA-A	c209	270	14, 1%	18, 1%	310	0, 37	0. 7 (0. 2-2. 5)
HLA-A	c212	270	20, 5%	19, 4%	310	0, 84	1. 1 (0. 3-3. 4)
HLA-A	c255	270	0, 0%	0, 0%	310	n. a.	n. a. (n. a. -n. a.)
HLA-A	c271	289	7, 8%	6, 1%	308	0, 60	1. 3 (0. 2-8. 1)
HLA-A	c295	292	80, 8%	53, 9%	310	7, 2E-05	3. 8 (1. 3-11. 5)
HLA-A	c213	292	1, 3%	7, 3%	309	0, 11	0. 2 (0. 0-6. 0)
HLA-A	c270	299	0, 0%	0, 0%	310	n. a.	n. a. (n. a. -n. a.)
HLA-A	c241	300	29, 5%	53, 0%	310	2, 9E-03	0. 4 (0. 2-1. 1)
HLA-A	c277	301	48, 1%	40, 1%	309	0, 20	1. 4 (0. 6-3. 6)
HLA-A	c264	301	51, 3%	44, 0%	310	0, 24	1. 4 (0. 6-3. 5)
HLA-A	c274	301	5, 1%	3, 0%	309	0, 27	2. 1 (0. 2-20. 5)
HLA-A	c214	302	9, 0%	8, 2%	310	0, 78	1. 1 (0. 2-5. 8)
HLA-A	c215	303	59, 0%	35, 5%	309	1, 0E-03	2. 6 (1. 0-6. 6)
HLA-A	c282	303	48, 7%	39, 0%	306	0, 13	1. 5 (0. 6-3. 9)
HLA-A	c259	303	20, 5%	19, 4%	310	0, 84	1. 1 (0. 3-3. 4)
HLA-A	c273	307	31, 6%	58, 2%	308	8, 1E-04	0. 4 (0. 1-1. 0)
HLA-A	c244	309	71, 4%	83, 1%	308	0, 055	0. 5 (0. 2-1.6mm)
HLA-A	c272	309	2, 6%	2, 6%	309	0, 96	1. 0 (0. 0-18. 7)

continued

GENE	PROBE	POS	EBV+	EBV-	NMISS	P	OR (99. 9% CI)
HLA-A	c267	311	20, 5%	19, 4%	310	0, 84	1. 1 (0. 3-3. 4)
HLA-A	c216	315	28, 2%	25, 9%	310	0, 59	1. 2 (0. 4-3. 3)
HLA-A	c349	363	26, 9%	53, 0%	310	7, 6E-04	0. 4 (0. 1-1. 0)
HLA-A	c317	363	0, 0%	0, 0%	310	n. a.	n. a. (n. a. -n. a.)
HLA-A	c395	365	84, 6%	61, 2%	310	2, 2E-04	3. 8 (1. 2-12. 4)
HLA-A	c396	368	18, 4%	24, 4%	306	0, 27	0. 7 (0. 2-2. 2)
HLA-A	c345	372	20, 5%	19, 4%	310	0, 84	1. 1 (0. 3-3. 4)
HLA-A	c380	376	0, 0%	0, 0%	310	n. a.	n. a. (n. a. -n. a.)
HLA-A	c363	399	94, 9%	84, 5%	310	0, 018	3. 8 (0. 6-24. 6)
HLA-A	c321	414	85, 9%	68, 8%	309	7, 4E-03	2. 7 (0. 8-9. 3)
HLA-A	c338	420	47, 4%	67, 2%	310	0, 011	0. 5 (0. 2-1. 2)
HLA-A	c368	423	7, 7%	6, 5%	309	0, 64	1. 3 (0. 2-7. 5)
HLA-A	c397	456	51, 3%	72, 8%	310	2, 5E-03	0. 4 (0. 2-1. 1)
HLA-A	c347	456	12, 8%	16, 4%	310	0, 48	0. 8 (0. 2-2. 8)
HLA-A	c348	506	34, 6%	60, 3%	310	6, 0E-04	0. 4 (0. 1-1. 0)
HLA-A	c361	506	25, 6%	24, 6%	310	0, 91	1. 0 (0. 4-3. 0)
HLA-A	c325	527	56, 4%	30, 2%	310	1, 4E-04	3. 0 (1. 2-7. 9)
HLA-A	c369	527	35, 9%	23, 7%	310	0, 048	1. 8 (0. 7-5. 0)
HLA-A	c324	528	7, 7%	6, 5%	310	0, 63	1. 3 (0. 2-7. 5)
HLA-A	c350	528	10, 3%	12, 6%	309	0, 75	0. 9 (0. 2-3. 8)
HLA-A	c326	530	16, 7%	18, 1%	310	0, 66	0. 8 (0. 3-2. 9)
HLA-A	c373	531	34, 6%	60, 3%	310	6, 0E-04	0. 4 (0. 1-1. 0)
HLA-A	c374	531	18, 0%	18, 5%	310	0, 97	1. 0 (0. 3-3. 3)
HLA-A	c339	532	48, 7%	71, 6%	307	2, 0E-03	0. 4 (0. 2-1. 1)
HLA-A	c328	535	1, 3%	0, 0%	310	1, 00	n. a. (n. a. -n. a.)
HLA-A	c340	542	68, 0%	78, 5%	310	0, 061	0. 5 (0. 2-1.6mm)
HLA-A	c330	545	26, 9%	29, 3%	310	0, 97	1. 0 (0. 4-2. 8)
HLA-A	c381	545	26, 9%	29, 3%	310	0, 97	1. 0 (0. 4-2. 8)

continued

GENE	PROBE	POS	EBV+	EBV-	NMISS	P	OR (99. 9% CI)
HLA-A	c351	561	35, 9%	23, 8%	309	0, 056	1. 8 (0. 7-4. 9)
HLA-A	c346	561	0, 0%	0, 0%	306	n. a.	n. a. (n. a. -n. a.)
HLA-A	c333	563	0, 0%	0, 0%	310	n. a.	n. a. (n. a. -n. a.)
HLA-A	c332	566	66, 7%	45, 9%	309	2, 0E-03	2. 5 (0. 9-6. 6)
HLA-A	c378	571	56, 4%	29, 9%	309	1, 3E-04	3. 1 (1. 2-8. 0)
HLA-A	c352	573	0, 0%	0, 0%	310	n. a.	n. a. (n. a. -n. a.)
HLA-A	c331	575	71, 8%	47, 0%	310	3, 4E-04	3. 0 (1. 1-8. 0)
HLA-A	c375	576	56, 4%	30, 2%	310	1, 4E-04	3. 0 (1. 2-7. 9)
HLA-A	c342	576	82, 1%	89, 7%	310	0, 15	0. 6 (0. 1-2. 1)

Chapter 6

Summary, Discussion and Future perspectives

Summary and Discussion

Classical Hodgkin lymphoma(cHL) is a typical malignant disease with a complex pathogenesis that is still not completely understood. In this thesis both environmental and genetic risk factors are explored in a Northern Chinese and in a Northern Dutch cHL population.

Epidemiology of cHL

CHL accounts for about 1% of all cancers and~30% of the lymphoid malignancies worldwide(1). Interestingly, a striking variation in the incidence of cHL has been observed between different geographic locations and/or racial groups (2). An almost 6-fold difference in the incidence of cHL was reported between Western and Oriental population groups with an incidence of 2. 3 and 0. 4 per 100, 000 inhabitants per year respectively (http://globocan. iarc. fr/). In addition, the age distribution pattern of cHL also differs between high and low incidence areas. In Western countries, it exhibits a distinctive bimodal pattern with one peak at young adult age and a second peak in the elderly (3, 4). In the few available studies in Asians, the first incidence peak is observed at a younger age, i. e. in adolescents, whereas the second peak is usually observed at the same age as in Western countries, with the exception of a single study from Japan in which a unique peak in the elderly was reported (5, 6). It should be noted that these studies are based on a limited number of patients varying from 23 to 70(7-9).

Infection with Epstein Barr Virus(EBV) was identified to play a causal role in the pathogenesis of cHL(10, 11). However, the frequency of EBV-associated cHL ranges from 30% in Caucasians to an intermediate frequency in Orientals to almost 100% in Hispanics (4, 12). EBV + cHL was always observed more frequently in children and elderly as compared to the other age groups regardless of the ethnic background(13, 14).

In chapter 2 of this thesis, we performed a large epidemiological study including 371 cHL patients from the northern part of China. We demonstrated both distinctive and consistent features when comparing the data with previous findings in the Asian population. The percentage of EBV + cHL patients was around 41% in the Northern Chinese patients, only a little bit higher than in Caucasians. However, a comparison between the Chinese and Dutch patient populations showed significant differences in terms of patients' age and gender distribution in relation to EBV status.

Expression of HLA antigens by cHL tumor cells in the Chinese cHL patients

Deficiency in the expression of Human leukocyte antigen(HLA) antigens by tumor cells is thought to be an important strategy for malignant cells to escape from host immunosurveillance(15, 16). This might be especially important in EBV+cHL, because EBV-derived antigenic peptides are known to consistently evoke immune responses. Previous studies performed in the Caucasian population have found that retention of HLA class I and HLA class II molecules is much more common in EBV+cHL as compared to EBV- cHL patients(17-19). Although there is no clear explanation for this paradox, the highly polymorphic nature of the HLA genes and the resultant functional variation between the HLA molecules might play a role. In addition, persistence of HLA class I expression is advantageous for the tumor cells to avoid Natural Killer cell-mediated lysis(20).

In chapter 3 we analyzed HLA class I and class II expression in a group of 145 Chinese cHL patients. In agreement with previous findings in the Caucasian population, membrane expression of HLA class I was strongly associated with positive EBV status in the Chinese cHL patients. In contrast, we found no significant association of membrane expression of HLA class II with EBV status in Chinese cHL. In Chinese EBV+cHL patients, HLA class II expression was lost

more often than in Caucasians(48% vs 30%). There is *in vitro* evidence that EBV infection and the related malignant transformation are controlled by CD4+T cells, depending on HLA class II restricted antigen presentation(21). Based on extreme differences in allele frequencies between racial groups, it can be hypothesized that HLA class II alleles that are prevalent in the Chinese population might effectively present EBV-derived peptides to the immune system, thereby exerting selection pressure to downregulate HLA class II expression.

Genetic associations between HLA alleles and cHL

The HLA complex, being an essential element of the human immune system, has been widely studied in various human diseases for its potential disease contribution(15, 16). Since virally infected cells can be strictly controlled by host immune surveillance, it is plausible to expect a difference in the HLA associations between EBV+ and EBV- cHL. Previous studies on HLA and cHL associations were mainly performed among Caucasians and significant effects of specific HLA genes were found in both sporadic and familial cHL patients (reviewed in (22)). Most of the above mentioned studies applied serological determination of usually only a few HLA antigens and EBV status was not taken into account. Each HLA serological type usually encompasses a group of distinct allele variants and the alleles of a serological group are functionally different in peptide binding and presentation (23). This implies that HLA association studies based on serology might give biased results. In addition, the extensive linkage disequilibrium (LD) in the HLA region between different HLA genes and between HLA genes and non-HLA genes might also affect association outcomes. Previous results from our research group based on a genetic screening analysis of the entire HLA region and subsequent detailed DNA based HLA-typing, showed that HLA-A1 is clearly overrepresented whereas HLA-A2 is underrepresented in EBV+cHL patients from the Northern part of the Netherlands(24).

Very recently, a study from Scandinavia and the United Kingdom showed that HLA-A1 homozygosity renders an approximately 4-fold increased risk of developing EBV+cHL, while HLA-A2 homozygosity predicts a 4-fold decreased risk(25).

HLA-A associations in the Chinese population

HLA-A*02 is the most common HLA-A allele in most populations worldwide. In Caucasians, HLA-A*02:01 is by far the most common allele variant and has been found to be a protective allele for the development of EBV+cHL(24, 25). In the Chinese population there are other HLA-A*02 allele variants with a high frequency in addition to HLA-A*02:01 such as HLA-A*02:06 and HLA-A*02:07 that are very infrequent in Caucasians (26). Notably, the HLA-A*02 subtypes differ in their capacity of peptide binding and presentation as well as the subsequent immune response(23). HLA-A*01 was found to be a risk allele for EBV+cHL in the Caucasian population, however HLA-A*01 is rare in the Chinese population. Because of the strong correlation between EBV + cHL and HLA-A*01 in Caucasian populations, one would expect that the proportion of EBV+cHL in the Chinese cHL patients would be lower. However, this proportion was consistently reported to be higher than in Caucasian patients(4, 12). Therefore HLA-A*01 is very unlikely to be a predominant susceptibility gene for EBV+cHL among the Chinese population. A possible minor effect still needs to be studied by determining the allele frequency of HLA-A*01 in the Chinese EBV+cHL patients.

In chapter 4 we analyzed the HLA-A*02 frequency in the Chinese population and observed similar frequencies in EBV+and EBV- cHL patients. Subsequent sequence based typing of the most common HLA-A*02 alleles revealed that HLA-A*02:07 is a risk factor for developing EBV+cHL in the Chinese population, whereas the other alleles are protective similar to the Western population. HLA-A*02:07 is a distinctive HLA-A*02 subtype that is almost exclusive to Orientals and has been found to confer a significant risk for the development of

Undifferentiated Nasopharyngeal Carcinoma (UNPC) in the southern Chinese population(27). A possible explanation for this finding was given by the less efficient presentation of EBV-derived peptides by HLA-A*02:07(28, 29). At least in the Chinese population, this allele can very well explain the distinctive geographic distribution of UNPC. These findings illustrate that HLA-linked ethnic diversity can explain geographical variation in disease incidence as is seen for cHL. In addition, it demonstrates that knowledge of the ethnic background is of particular importance for genetic association studies.

HLA-based genetic susceptibility to cHL in the Dutch population

In chapter 5 of this thesis, we studied a group of Dutch cHL patients(n=338) by detailed DNA-based HLA typing. HLA-B51 and HLA-DR7 were found to be a susceptible and a protective HLA allele for cHL irrespective of the EBV status. Three class II associations were observed in the EBV- cHL population with an increase of HLA-DR15(2) and a decrease of HLA-DR4 and HLA-DR7. Besides confirmation of the strong genetic effects of HLA-A1 and HLA-A2 in EBV+cHL patients, we also found an association of HLA-DR10 and HLA-B37 with EBV+ cHL. It should be noted that these alleles are in linkage disequilibrium with HLA-A *01. The allele frequency of HLA-DR11(5) was significantly decreased in the EBV +cHL population. SSOP analysis revealed significant differences between EBV+ and EBV-cHL patients for 19 probes that discriminate between HLA-A*01 and HLA-A*02. In conclusion, the HLA-A1 and HLA-A2 antigens and not a specific single nucleotide variant shared by multiple alleles are responsible for the association with EBV + cHL. The differential HLA-based genetic background between the EBV+and EBV-cHL suggests that an undefined infectious agent other than EBV or a(group of) specific tumor-associated antigen(s) is involved in the pathogenesis of EBV- cHL.

In conclusion

In this thesis we showed marked differences in cHL epidemiology between Caucasian and Oriental populations. The complex nature of the disease may at least be partially explained by differences in genetic background, which is most notably in the HLA genes.

Future perspectives

In this thesis, we describe the epidemiological features of a group of cHL patients from Northern China in which both similarities and differences were found in comparison to the published data. However, as there is only limited data regarding the epidemiological features of cHL among Asians, it is worthwhile to identify and study more cHL patients to further illustrate the similarities and differences. Moreover, it should be noted that the previous studies reported on the epidemiological features of cHL in Chinese subpopulations from Hong Kong and Taiwan, consisting mainly of descendants from the Southern Chinese population who are genetically different from the Northern Chinese population. Given the large differences in the frequency distribution of HLA alleles between Southern and Northern Chinese populations, it would be of great value to separately analyze and compare cHL patients from Southern and Northern China.

Currently, a strong association between tumor cell membrane expression of HLA class I and EBV+cHL was observed in both Caucasians and Chinese cHL patients. However, we did not find an association of membrane expression of HLA class II with EBV+cHL in the Chinese population. Our group previously reported that in the Dutch population, absence of HLA class II expression was associated with adverse outcome(30). We found a relatively higher frequency of loss of HLA class II expression in the Chinese cHL patients compared to the Dutch population(46. 2% expression vs 58. 6% expression). Whether the prognostic

significance of HLA class II expression in cHL would also be present in the Chinese population needs to be studied. Three microsatellite markers within the HLA class II region have been reported to be in close relationship with HLA class II expression in cHL in the Dutch population(30). One or more specific HLA alleles could be involved, but this association could also be caused by transcriptional regulation sites or a non-HLA gene. In the future, a fine screening of the class II region should be done to identify which genetic locus or loci account(s) for this association. Linking of the HLA typing data to the expression of HLA class II molecules may be helpful for illuminating the above association. Ideally, as a next step we should study these HLA class II alleles in combination with HLA class II downregulation in the Chinese population.

Due to differences in the distribution of common and well documented HLA alleles between different population groups, it can be expected that different HLA-linked genetic associations with certain diseases can be found in different ethnic groups. We found that the ethnic difference in the distribution of HLA-A*02 subtypes alters the association between HLA-A*02 and EBV+cHL. In addition to HLA-A*02, HLA-A*01 is also very common in the Caucasian population, which has been identified to confer a significant risk for EBV + cHL. Although the frequency of HLA-A*01 is rather low in the Orientals, its frequency in the Chinese EBV+cHL patients should be explored since it may be an additional risk factor to HLA-A*02:07. Some other HLA alleles such as HLA-A*11 are common in the Chinese population but not in the Caucasians. Notably, an effective anti-EBV immune response can be elicited in the context of HLA-A*11 and this may very well explain the protective effect of HLA-A*11 in UNPC(31). Therefore, it would be ideal to perform a detailed screening of the HLA genes that are involved in the pathogenesis of cHL in the Chinese population. This would be of particular benefit for better understanding the genetic variation of cHL between the Dutch and Chinese populations. A small proportion of the Dutch EBV+cHL patients possess

the HLA-A*02 allele and it is unclear how the EBV positive tumor cells escape from an effective anti-EBV cytotoxic T-lymphocyte immune response. It might be hypothesized that the HLA-A*02 allele is no longer functional in the tumor cells of these patients. HLA-A*02 expression might be decreased or completely lost on the cell surface of the tumor cells. This could be studied by immunohistochemistry using a HLA-A*02-specific antibody. In addition, the peptide-binding cleft of the HLA molecule, encoded by exons 2 and 3 of the gene, determines the characteristics of presented peptide and hence influences the immune response. Therefore, we plan to sequence exons 2 and 3 of the HLA-A*02 gene in Dutch EBV+cHL patients to screen for putative mutations and/or deletions that could alter the peptide-binding affinity and/or specificity.

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Nederlandse samenvatting

Achtergrond

Hodgkin lymfoom is een vorm van B cel kanker die wordt gekarakteriseerd door een opvallende minderheid van tumorcellen in een uitgesproken achtergrond van reactieve cellen. Het Epstein-Barr virus (EBV) wordt in de tumorcellen van een variabel percentage(20-70%) van de pati nten aangetroffen en dit percentage is afhankelijk van de etnische achtergrond van de pati nten. De incidentie van HL varieert per geografische locatie en etniciteit, en is in de westerse bevolking 2, 3 per 100. 000 inwoners per jaar. Opmerkelijk is dat ook de leeftijdsverdeling verschilt per populatie. In de westerse bevolking wordt EBV+HL m. n. in kinderen / pubers en ouderen aangetroffen.

De meest voorkomende variant is het klassiek Hodgkin lymfoom. Er wordt al jarenlang veel onderzoek gedaan naar de ontstaanswijze van klassiek Hodgkin lymfoom en op basis van deze studies weten we dat er omgevings- en genetische componenten zijn. In het geval van EBV + HL valt er een afweerreactie te verwachten tegen eiwitten(peptiden) afkomstig van het EBV. Dit gebeurt echter niet en in combinatie met de grote overmaat aan reactieve cellen in de directe omgeving van de tumorcellen is dit op zijn minst onverwacht te noemen. Een mogelijke oorzaak zou kunnen zijn dat de expressie van HLA, het molecuul dat peptiden van het EBV aan het afweersysteem kan presenteren, verloren gaat. In HL wordt inderdaad vrij vaak verlies van expressie van HLA gevonden op de tumorcellen, maar opvallend genoeg vooral in EBV- HL en veel minder vaak in EBV+ HL. In dit proefschrift hebben we een populatie van klassieke Hodgkin lymfoom pati nten onderzocht uit het noorden van China m. b. t. leeftijd en EBV verdeling, HLA klasse I expressie in relatie tot EBV en genetische associatie met HLA-A allelen. Daarnaast is een populatie van klassiek Hodgkin lymfoom pati nten uit noord Nederland onderzocht m. b. t. HLA allel frequenties d. m. v. DNA gebaseerde HLA subtypering.

Epidemiologie van cHL

In hoofdstuk 2 van dit proefschrift hebben we een epidemiologische studie gedaan in 371 Chinese klassiek HL pati nten en deze populatie vergeleken met de Nederlandse populatie. Het percentage EBV positiviteit was 41%, dit is iets hoger dan het gemiddelde in de westerse populatie. De incidentie curve voor EBV +and EBV- klassiek HL liet duidelijke verschillen zien tussen de Chinese en de Nederlandse populatie m. b. t. leeftijdsverdeling en geslacht.

HLA expressie en allelen in Chinese cHL pati nten

In hoofdstuk 3 hebben we expressie van HLA klasse I en II onderzocht in 145 Chinese klassieke HL pati nten. In overeenstemming met eerder gepubliceerde data in de westerse bevolking vonden we dat EBV+pati nten meestal HLA klasse I positief waren en dat EBV- pati nten relatief vaak expressie van HLA klasse I hadden verloren. Voor HLA klasse II zagen we geen significante verschillen tussen EBV- en EBV+pati nten, in tegenstelling tot eerder gepubliceerde data in de westerse bevolking. Aangezien het HLA allel bepaalt welke peptiden worden gepresenteerd en daarmee dus ook hoe effectief het afweersysteem op bepaalde peptiden reageert, kan het verschil tussen de populaties verklaard worden door bekende etnische verschillen in HLA allelfrequenties. In de westerse populatie is aangetoond dat bepaalde HLA-A types(waarbij elk type een familie is van allelen) geassocieerd zijn met EBV+HL. Het HLA-A1 type is een risicofactor voor het ontwikkelen van EBV+HL, terwijl het HLA-A2 type juist beschermend werkt. Deze associatie kan goed verklaard worden doordat individuen met het HLA-A2 type goed in staat zijn om een effectieve afweerreactie op te wekken tegen EBV afkomstige peptiden, terwijl individuen met het HLA-A1 type dat juist niet kunnen.

In een vervolgonderzoek hebben we gekeken naar de frequentie van het HLA-A2 type in de Chinese HL populatie. Het HLA-A1 type is niet onderzocht

aangezien dit allel heel erg zeldzaam is in de Chinese populatie. In hoofdstuk 4 hebben we aangetoond dat de HLA-A2 frequentie in de EBV+HL populatie niet verschilt van EBV- HL en een controle populatie. Aangezien ook de HLA-A2 subtype(of allel-) frequenties nogal verschillen tussen de westerse en Chinese populatie hebben we vervolgens een "sequence based subtyping" gedaan. Hieruit kwam dat de frequentie van het HLA-A*02:07 allel aanzienlijk verhoogd was en de frequentie van alle andere HLA-A2 allelen juist sterk verlaagd in EBV+HL t. o. v. EBV- en controles. Dit verschil kan mogelijk verklaard worden doordat individuen met het HLA-A*02:07 allel ook geen effectieve afweerreactie op EBV kunnen induceren, vergelijkbaar met het HLA-A1 type in de westerse populatie.

HLA typering in de Nederlandse klassieke HL populatie

In hoofdstuk 5 van dit proefschrift hebben we in 338 Nederlandse HL pati nten een HLA typering gedaan m. b. v. een op PCR gebaseerde "sequence specific oligonucleotide probe"(SSOP) techniek. Als controlegroep hebben meer dan 5. 000 bloedbank controles gebruikt. HLA-DR7 was significant verlaagd en HLA-B51 significant verhoogd in de HL populatie t. o. v. controles. In de EBV- HL pati nten vonden we een sterke associatie met drie HLA klasse II genen. De frequentie van HLA-DR15(2) was verhoogd en die van HLA-DR4 en HLA-DR7 verlaagd. In EBV+ HL pati nten vonden we naast de bekende associatie met HLA-A een significante toename van de frequenties voor HLA-B37 en HLA-DR10 en een verlaging van de HLA-DR11(5) frequentie. Analyse van de individuele SSOPs lieten significante verschillen zien tussen EBV+en EBV- HL pati nten voor 19 van de 358 probes. De specificiteit van deze 19 probes corresponderen met HLA-A1 en HLA-A2. Op basis van deze studie kunnen we concluderen dat er verschillende genetische associaties zijn voor EBV- en EBV+HL. Daarnaast zijn er ook twee associaties die aanwezig zijn in zowel EBV+alsook in EBV- HL pati nten.

Samenvattend

In dit proefschrift hebben we aangetoond dat er verschillen zijn in epidemiologie, EBV, leeftijd en HLA associatie tussen de Chinese en Nederlandse HL pati nten. Daarnaast hebben we in de Nederlandse populatie aangetoond dat er zowel verschillen als overeenkomsten zijn tussen EBV+en EBV- HL pati nten m. b. t. genetische HLA associaties.

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